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EFFECTS OF AMPHENONE B AND OF DIET ON CHOLESTEROL LEVELS IN THE RAT[1]

K. K. CARROLL

Abstract

Amphenone B was added in concentrations of 0.1 to 0.5% to a number of synthetic diets and to a fox chow diet. These were then fed to young male rats to determine the effects on adrenal weight and on the cholesterol content of the adrenals, liver, and plasma. The rats fed amphenone mixed with synthetic diets showed a greater increase in adrenal size and adrenal cholesterol than those fed amphenone mixed with the chow diet. The liver and plasma cholesterol values of rats fed amphenone on synthetic diets were also increased appreciably while those of rats fed amphenone in the chow diet were near normal. Synthetic diets containing erucic acid and amphenone appeared to have the greatest effect on adrenal size and cholesterol content but other synthetic diets in some cases caused a greater increase in liver and plasma cholesterol concentrations. An attempt to examine the effect of injected amphenone on cholesterol levels in rats fed different diets was limited by the fact that high doses of amphenone cause prolonged anesthesia.

Introduction

The work of Hertz and his associates has disclosed a number of interesting biological effects of the compound amphenone B. These effects include progestational changes in the endometrium of the rabbit (1), an atypical folliculoid action on the endometrium of the ovariectomized female rat, enlargement of the thyroid, adrenal enlargement with accumulation of cholesterol, and general anesthesia at high dose levels (2, 3). The folliculoid action has been shown to occur both in the hypophysectomized and in the adrenalectomized rat, and this suggests that the effect is due to a direct trophic action of amphenone on the uterus and vagina. The effects on the adrenal and thyroid require the presence of an intact pituitary and it is presumed that amphenone causes adrenal and thyroid hypertrophy by inhibiting the formation or secretion of their respective hormones, thus causing increased secretion of thyrotropin or corticotropin (4). Experimental work has shown that amphenone does indeed inhibit the output of adrenal cortical hormones, both in experimental animals (5–7) and in humans (8–10), although Vogt observed the inhibition in rats only when amphenone was given intravenously and not when it was administered orally (11, 12).

[1]Manuscript received May 12, 1959.
Contribution from The Collip Medical Research Laboratory, University of Western Ontario, London, Ontario.

The effects of amphenone on the adrenal cortex were of particular interest to us because work in our laboratory had shown that an accumulation of cholesterol in the adrenal cortex occurred in rats fed long-chain fatty acids such as erucic acid and nervonic acid (13). This action of fatty acids was also dependent on the presence of the pituitary but no evidence was obtained of altered adrenal function (14). The concentration of cholesterol in the liver and the amount excreted in the feces were also increased in rats fed erucic acid (15). The present experiments were undertaken to compare the effects of amphenone and of erucic acid on cholesterol metabolism in the rat and to determine whether their effects were additive.

Experimental

Male Sprague–Dawley rats weighing 80–90 g were caged in groups of three and used for the feeding experiments. Amphenone B, in the form of the dihydrochloride, was added at various concentrations to a powdered fox chow diet (Master Meal[2]) and to a number of synthetic diets, containing either no fat or 15% by weight of various individual fatty acids. The synthetic diets had the following composition, expressed as parts by weight: fat-free diet—casein (purified) 18, dextrose 72, Phillips-Hart salt mixture 4, and cellu flour 5, while the fat-containing diets consisted of casein 22, dextrose 52, fatty acid 15, salt mixture 5, and cellu flour 5. A mixture of water-soluble vitamins was added to each synthetic diet at the rate of 15 ml per kg. The source of dietary materials and the composition of the vitamin mixture have been described previously (16). The amphenone B was generously provided by CIBA of Canada Limited, Montreal, through the co-operation of Dr. C. Walter Murphy. It should be noted that this compound has recently been assigned the new structural formula shown in Fig. 1 (17). The amphenone B was dissolved in water (50 mg per ml) for the experiments in which it was injected intraperitoneally.

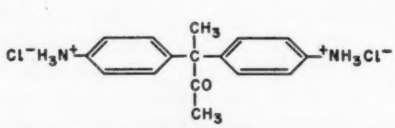

FIG. 1. Amphenone B dihydrochloride.

At the conclusion of the feeding experiments, the rats were anesthetized with Nembutal and a sample of blood was obtained by heart puncture. Heparin was used to prevent clotting. The blood was centrifuged and the cholesterol content of the plasma determined by the Sperry–Webb procedure (18). Individual adrenals were placed in 15-ml centrifuge tubes containing 3 ml of acetone–alcohol (1:1) and a few grains of washed sand. They were crushed with a stirring rod and later extracted with three 3-ml portions of boiling acetone–alcohol. The extracts were filtered, made to 10 ml, and aliquots (5 ml for free and 0.5 to 2 ml for total) were used for cholesterol determinations by the

[2]Master Fox Breeder Starter Ration, Toronto Elevators, Ltd., Toronto.

Sperry–Webb procedure. Samples of liver weighing approximately four grams were ground in a mortar with acetone–alcohol and transferred to 50-ml Erlenmeyer flasks. They were then extracted with three 15-ml portions of boiling acetone–alcohol. The extracts were filtered, made to 50 ml, and aliquots (0.5 to 1 ml) were used for cholesterol determinations. In experiments in which fecal cholesterol was measured, the feces were collected for 7-day periods as in previous experiments (15). They were dried, thoroughly mixed, powdered, and 100-mg aliquots were extracted with three 3-ml portions of boiling acetone–alcohol. The extracts were filtered, made to 10 ml, and 3-ml aliquots were used for cholesterol determinations.

Results

The results in Table I illustrate the impressive effect of amphenone in increasing the weight and cholesterol content of the adrenals. The effect was greater with a diet containing erucic acid than with one containing oleic acid when amphenone was added at concentrations of 0.1 or 0.25%, but there was no significant difference between the results obtained with the two diets when amphenone was added at a 0.5% concentration. In other experiments, amphenone was fed at a concentration of 0.25% in synthetic diets containing no fat

TABLE I

Effect of amphenone on adrenal weight and cholesterol content

Type of diet	Amphenone concentration in diet %	Daily* food intake (g)	Days on diet	Number of rats	Body† weight (g)	Adrenal weight (mg)	Adrenal cholesterol	
							Free, % of wet wt.	Total, % of wet wt.
15% erucic acid	0	14	20	6	153±5.7‡	35±1.4‡	0.7	10.4±0.5‡
	0.1	16	21	8	140±2.7	46±2.3	0.6	17.4±1.8
	0.25	14	21	6	136±3.5	61±2.9	0.8	18.5±0.6
	0.5	9	21	6	101±8.8	82±8.6	1.4	22.1±1.2
15% oleic acid	0	14	20	6	189±4.1	33±0.9	0.6	6.0±0.2
	0.1	13	22	9	184±5.7	38±1.4	0.5	7.7±0.3
	0.25	12	21	6	147±3.9	48±3.2	0.5	14.7±0.9
	0.5	9	21	6	113±2.2	75±3.9	1.5	22.9±1.2
Fat-free	0.25	15	21	5	139±8.8	60±3.0	0.7	17.6±0.7
15% palmitic acid	0.25	15	21	6	141±3.9	50±4.0	0.7	15.1±1.3
Master Meal	0	17	20	6	185±6.2	35±1.3	0.6	4.3±0.7
	0.25	17	21	6	152±2.8	39±2.0	0.6	10.1±1.1
	0.5	12	21	6	128±6.7	62±3.6	0.8	15.4±0.7
15% oleic acid	0.25	11	14	6	136±3.4	42±1.3	0.6	13.8±0.3
	0.25	11	7	6	113±3.4	41±3.1	0.5	11.4±0.7
	0.25	12	3	6	153±2.0	30±0.8	0.5	5.6±0.5

*The food intake was corrected for spillage.
†The average starting weight was 80–90 g except in the 3-day experiment where it was 144 g.
‡Standard error of the mean.

or 15% palmitic acid and the effects on the adrenal were intermediate to those obtained with the diets containing erucic or oleic acids. When amphenone was fed with Master Meal, the increases in adrenal weight and cholesterol content which resulted were less than those observed when amphenone was fed with any of the synthetic diets. From these experiments it appears that feeding a combination of erucic acid and amphenone has a greater effect on adrenal

cholesterol than feeding either one alone, but it is difficult to determine whether their effects are strictly additive because of the experimental variation in the results. Erucic acid alone has little effect on adrenal weight but in combination with amphenone it appears to enhance the effect of the latter on adrenal weight increase as well as on adrenal cholesterol concentration.

The figures for body weight given in Table I show that amphenone has a retarding effect on weight gain in the rat, particularly at the higher dose levels. The food intake was also much lower when diets containing 0.5% amphenone were fed.

Erucic acid had been shown in previous experiments to increase the concentration of cholesterol in the liver and the rate of cholesterol excretion in the feces (15). Therefore, liver and fecal cholesterols were measured in the experiments reported in Table I, and plasma cholesterols were determined as well. The results are presented in Table II.

TABLE II

Effect of amphenone on liver, plasma, and fecal cholesterol

Type of diet	Amphenone concentration in diet %	Days on diet	Liver weight (g)	Liver cholesterol		Plasma cholesterol		Fecal cholesterol*	
				Free	Total	Free	Total	Free	Total
				(% of wet weight)		(mg/100 ml)		(mg/rat/day)	
15% erucic acid	0	20	8.1±0.4†	0.26	0.43±0.02†	19±2†	87±6†	11.2	26.7 (4)
	0.1	21	7.9±0.2	0.27	0.70±0.04	46±4	192±13	—	—
	0.25	21	9.6±0.6	0.20	0.46±0.06	45±5	165±20	4.8	15.5 (2)
	0.5	21	8.4±0.5	0.25	0.50±0.04	69±4	196±16	—	—
15% oleic acid	0	20	10.3±0.4	0.23	0.40±0.01	30±1	111±3	9.6	10.9 (4)
	0.1	22	11.3±0.7	0.24	0.55±0.04	26±3	109±35	—	—
	0.25	21	11.5±0.5	0.22	0.63±0.06	48±4	172±9	7.0	7.9 (4)
	0.5	21	9.2±0.4	0.23	0.73±0.04	49±2	158±12	—	—
Fat free	0.25	21	7.8±0.6	0.21	0.35±0.03	40±3	138±6	3.8	3.5 (2)
15% palmitic acid	0.25	21	8.7±0.3	0.22	0.36±0.03	57±7	201±19	1.9	10.9 (2)
Master Meal	0	20	7.7±0.3	0.25	0.31±0.01	23±1	83±5	17.8	19.0 (4)
	0.25	21	9.9±1.1	0.17	0.23±0.02	26±5	72±11	—	—
	0.5	21	10.4±0.6	0.23	0.28±0.03	39±3	107±5	—	—
15% oleic acid	0.25	14	11.7±0.5	0.20	0.46±0.03	50±5	163±11	—	—
	0.25	7	10.0±0.6	0.23	0.55±0.04	61±5	194±11	—	—
	0.25	3	11.2±0.2	0.22	0.38±0.02	45±3	138±5	—	—

*The number of determinations is shown by the figures in parentheses.
†Standard error of the mean.

In the absence of amphenone, the concentration of cholesterol in the liver was significantly higher in rats fed synthetic diets containing erucic or oleic acid than that in the liver of rats fed Master Meal. The plasma cholesterol was also slightly increased in rats fed the diet containing oleic acid. When amphenone was mixed with these two synthetic diets and fed to rats, the plasma cholesterol levels were increased appreciably and the liver cholesterol concentrations were higher than those observed in the absence of amphenone. The plasma cholesterol did not increase progressively as the amount of amphenone in the diet was increased, but values obtained after feeding 0.25% or 0.5% amphenone in synthetic diets were generally in the range of 140 to 200 mg per 100 ml. In contrast to these results, the feeding of amphenone in a diet of Master Meal had relatively little effect on the concentration of plasma

cholesterol and showed no tendency to increase the concentration of cholesterol in the liver.

The effect on cholesterol levels of length of time on the diet was investigated with a synthetic diet containing 15% oleic acid with 0.25% amphenone. An increase in plasma and liver cholesterol values was observed as early as 3 days after the animals were placed on this diet. In the results obtained with synthetic diets, most of the increase in liver and plasma cholesterol occurred in the ester cholesterol fraction, but the free cholesterol content of plasma was definitely increased in most experiments in which amphenone was fed.

In the experiments in which fecal cholesterol was measured there was no indication that amphenone increased the rate of excretion. In fact, the figures suggest that it may do the reverse, although more determinations would be required to establish the significance of these results. The synthetic diets used in these experiments were essentially free of sterol whereas the Master Meal contained chromogenic sterol. Fecal sterols were therefore not measured in the experiments in which amphenone was added to Master Meal.

The difference between the effect on liver and plasma cholesterol of rats fed amphenone in synthetic diets and that obtained in rats fed amphenone in the chow diet is very striking, and it seemed of interest to determine whether this difference could also be observed when the amphenone was administered parenterally. Some further experiments were therefore carried out in which amphenone was given once daily by intraperitoneal injection to rats maintained on Master Meal or on a synthetic diet containing erucic acid. The effects of this treatment on cholesterol levels are shown in Table III. Amphenone given

TABLE III

Effect of injected amphenone

				Total cholesterol		
Type of diet	Amphenone given (mg/day)	Body weight	Adrenal weight	Adrenal (% of wet wt.)	Liver (% of wet wt.)	Plasma (mg/100 ml)
15% erucic acid	15	113 ± 10*	60 ± 0.4*	20.6 ± 1.1*	0.37 ± 0.02*	86 ± 1*
Master Meal	15	185 ± 12	39 ± 1.5	10.5 ± 1.7	0.23 ± 0.01	71 ± 4
Master Meal	25	160	44.6	13.4	0.26	51

*Standard error of the mean.

NOTE: Each group contained three rats but one rat died in the group given 25 mg per day of amphenone. The injections were given daily for 21 days.

in this way caused no increase in liver or plasma cholesterol concentrations of rats fed either Master Meal or the synthetic diet. It was not practicable to use amounts of amphenone greater than 25 mg per injection because the rats became anesthetized for prolonged periods.

Discussion

The feeding of erucic acid to rats has previously been shown to cause an increase in the concentration of adrenal and liver cholesterol and an increased excretion of cholesterol in the feces. The administration of amphenone has also been found to increase adrenal cholesterol concentration but neither erucic acid nor amphenone have been shown to affect blood cholesterol levels (15, 19).

It was therefore of considerable interest to find that the concentration of plasma cholesterol was markedly increased in rats fed a diet containing both erucic acid and amphenone. Further investigation showed that this effect could also be produced by feeding amphenone in a number of different synthetic diets which did not contain erucic acid. However, very little increase in plasma cholesterol was observed when amphenone was fed with a fox chow diet. High liver cholesterol concentrations were found in rats fed synthetic diets containing amphenone while normal values were found in rats fed amphenone mixed with the chow diet.

It is not apparent from the results why the effects of amphenone fed in synthetic diets should be different from those of amphenone fed in a chow diet. When amphenone is administered by mouth, as in these experiments, the nature of the diet and the food intake affect the amount received by the animals. An attempt to eliminate this factor by giving the amphenone intraperitoneally was limited by the amount of amphenone which could be administered in this way. Further experiments are being carried out in an attempt to clarify the nature of this effect of diet.

The findings of this study illustrate the fact that a small amount of a dietary component unrelated in structure to cholesterol may have an important effect on liver and plasma cholesterol levels. The results also show that the magnitude of the effect may be greatly modified by other components of the diet.

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THE RELATION BETWEEN DRUG-INDUCED EXCITATION IN THE CAT AND ADDICTION LIABILITY[1]

H. CULLUMBINE[2] AND T. S. KONOP

Abstract

The production of central excitation in the cat by various opium alkaloids, some synthetic analgesics, and related compounds has been studied. Dihydromorphinone, diamorphine, ketobemidone, levorphan, dipipanone, methadone, apomorphine, morphine, dihydrocodeinone, dihydrocodeine, phenadoxone, alphaprodine, codeine, meperidine, dionine, apocodeine, and pholcodine were all found to cause excitation when injected subcutaneously into cats.

Papaverine, narcotine, cotarnine, hydrocotarnine, dextromethorphan, persedon, nalorphine, and levallorphan did not produce excitation in the cat. Prior administration of nalorphine or levallorphan prevented the production of excitation by morphine.

Tolerance to the excitatory effect of morphine in the cat can be rapidly established.

It is concluded that there is a relationship, although not an exact one, between the ability to produce excitation in the cat and the danger of producing addiction in man by the opium alkaloids and many synthetic analgesics.

Mayor (1), in 1908, claimed that only those opium alkaloids which produced central excitation in the cat would be likely to cause addiction in man. Akad (2) found some parallelism between ease of production of excitation in the cat and the development of addiction in man for dihydrohydroxycodeinone (Eucodal), dihydrocodeinone (Dicodide), dihydromorphinone (Dilaudid), diamorphine (Heroine), meperidine (Demerol), and ethylmorphine (Dionine). More recently, Tavat and Akcasu (3) have reported that morphine, codeine, racemorphan, ketobemidone, methadone, and meperidine, which are known to produce addiction in man, all caused excitation in the cat, and that the greater the addiction-producing potency of a drug then the smaller was the dose of that drug which was required to produce the exciting effect. Dextromethorphan, which is said not to cause addiction in man, had no exciting effect on the cat. Neither did levallorphan, which was shown to prevent the exciting effect of morphine.

Therefore there would appear to be a relationship between the production of central excitation in the cat and the danger of producing addiction in man by the opium alkaloids and many synthetic analgesics. There may exist here the basis of a simple laboratory method, quick and with some quantitative basis, for predicting the possibility of addiction occurring in man following the use of analgesics. With this in view, an attempt has been made to repeat and to expand the observations of the previous investigators.

Method

The procedure used was similar to that employed by Tavat and Akcasu (3). Adult cats of either sex were injected subcutaneously with the drug being

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studied. The cat was then placed in a wire cage of sufficient size to allow the cat free movement. The cage was suspended from a hook by a spring and the up-and-down movements of the spring, caused by the activity of the cat in the cage, were recorded on slowly moving smoked paper. The experiments were conducted in an isolated, quiet room so as to minimize extraneous causes of excitement. The following drugs were tested:

(*a*) The alkaloids of opium: morphine sulphate, codeine sulphate, papaverine hydrochloride, narcotine hydrochloride, cotarnine hydrochloride, hydrocotarnine hydrochloride.

(*b*) The morphine derivatives: diamorphine hydrochloride, ethylmorphine hydrochloride, dihydromorphinone hydrochloride, dihydrocodeinone bitartrate, dihydrocodeine bitartrate, apomorphine hydrochloride, apocodeine hydrochloride.

(*c*) The synthetic substances: meperidine hydrochloride (ethyl-1-methyl-4-phenylpiperidine-4-carboxylate), methadone hydrochloride (*dl*-6-dimethylamino-4,4-diphenyl-3-heptonone), alphaprodine hydrochloride (*dl*-α-1,3-dimethyl-4-phenyl-4-propionoxy piperidine hydrochloride), ketobemidone hydrochloride (4-(*m*-hydroxyphenyl)-1-methyl-4-piperidyl ethyl ketone), levorphan tartrate (*l*-3-hydroxy-N-methyl-morphinan tartrate), dextromethorphan hydrobromide (*d*-3-methoxy-N-methyl-morphinone hydrobromide), persedon (3,3-diethyl-2,4-diketo-tetrahydropyridine), pholcodine sulphate (morpholinylethylmorphine sulphate), dipipanone (4,4-diphenyl-6-piperdine-3-heptanone hydrochloride), phenadoxone (6′-morpholino-4,4-diphenylheptan-3-one).

(*d*) The morphine antagonists: nalorphine (N-allyl-normorphine hydrobromide) and levallorphan tartrate (*l*-3-hydroxy-N-allylmorphinan tartrate).

The dose of each drug was varied until the minimum dose required to produce motor excitement was found. Cats were observed for a period of 4 hours before it was decided that no excitement had been caused by a particular dose. If any cat was repeatedly used, then an interval of several days was allowed to elapse between each injection in order to avoid the development of tolerance with alteration of the threshold dosage for excitement.

Results

Of the analgesics tested, heroine, dihydromorphinone, ketobemidone, levorphan, dipipanone, methadone, apomorphine, morphine, dicodid, dihydrocodeine, phenadoxone, alphaprodine, codeine, meperidine, dionine, apocodeine, and pholcodine all caused excitation when injected subcutaneously into cats, and the order in which the drugs are listed above indicates the order of increasing dosage required to produce this excitation. The minimum dose of each drug required to cause excitation is shown in Table I.

The drugs which did not cause excitation when given in high doses were papaverine hydrochloride (128 mg/kg), narcotine hydrochloride (64 mg/kg), cotarnine hydrochloride (80 mg/kg), hydrocotarnine hydrochloride (80 mg/kg), persedon (150 mg/kg), dextromethorphan hydrobromide (80 mg/kg), nalorphine hydrobromide (60 mg/kg), and levallorphan tartrate (40 mg/kg).

TABLE I

Minimum cat exciting dose and index of addiction liability for certain narcotic analgesics

Drug	Exciting dose in cat, mg/kg	Index* of addiction
Diamorphine HCl	0.1	25
Dihydromorphinone HCl	0.2	10
Ketobemidone HCl	0.25	32
Levorphan HCl	1.0	2
Dipipanone HCl	1.0	20
Methadone HCl	1.5	3.3
Apomorphine HCl	1.5	3.3
Morphine SO_4	2.0	4
Dihydrocodeinone bitartrate	2.4	0.4
Dihydrocodeine bitartrate	3.0	3.3
Phenadoxone HCl	3.6	6.9
Alphaprodine HCl	4.0	10
Codeine SO_4	5.0	3
Meperidine HCl	32.0	0.8
Ethylmorphine HCl	38.4	0.4
Apocodeine HCl	40.0	0.5
Pholcodine SO_4	120.0	0.008

*Index of addiction liability $= \frac{\text{minimum therapeutic dose (mg/kg) in man}}{\text{minimum exciting dose (mg/kg) in cat}}$.

The observation of Tavat and Akcasu (3) that levallorphan would prevent the exciting action of morphine was confirmed (e.g. 8 mg/kg morphine sulphate given after 10 mg/kg levallorphan tartrate had no stimulating action). A similar protective effect was also noted for nalorphine.

The cat quickly becomes tolerant to the exciting effect of morphine. The daily injection of 1 mg/kg morphine sulphate for 3 or 4 days increases the minimum dose of morphine required for excitation. After daily morphine for a more prolonged period (e.g. 1 mg/kg for 2 to 3 weeks) as much as 35 mg/kg morphine sulphate can be administered without producing excitation.

Discussion

Of the drugs found to cause excitation, heroine, dihydromorphinone, ketobemidone, levorphan, dipipanone, methadone, morphine, dihydrocodeinone, phenadoxone, alphaprodine, codeine, meperidine, and dionine are known to be addicting. Conversely, addiction has not been reported from those drugs which did not cause excitement.

Tavat and Akcusu (3) claim that the danger of producing addiction by the use of a given drug is directly related to the ease with which that drug can produce excitation in the cat. Our results do not always confirm this.

The exciting dose of heroine is less than that of morphine; this dose of morphine in turn is less than the required dose of dihydrocodeinone and the latter's dose is smaller than that of codeine. Similarly, the exciting dose of morphine or of ketobemidone is less than that of alphaprodine, which dose is smaller than that of meperidine. A similar relative order of liability to produce addiction has been noted for these drugs in man (Himmelsbach (4), Seevers and Pfeiffer (5), Small, Eddy, Mossettig, and Himmelsbach (6), Isbell (7)).

The order of addiction liability in the monkey has been reported to be heroine, morphine, dihydromorphinone, and codeine (8). This order is the same as that found in man but in our experiments on cats, dihydromorphinone should be more addicting than morphine.

Dihydromorphinone is said to show an addiction liability which is intermediate between that of morphine and codeine (Seevers and Pfeiffer (5)) but the exciting dose of morphine is 10 times that of dihydromorphinone. Similarly, meperidine and methadone are shown by Vogel, Isbell, and Chapman (9) to be situated between that of morphine and codeine. However, our results indicate that methadone should be more addicting than morphine, and that meperidine should be less addicting than codeine. Further, ketobemidone and morphine have a similar potency in producing euphoria (Isbell (7)) but their exciting doses show an eightfold difference.

Mayor (1) assessed the addicting liability of drugs by calculating the ratio of the minimum therapeutic dose (mg/kg) in man and the minimum dose (mg/kg) required to cause excitation in the cat. We have used our results to calculate a similar ratio, which could be called the "index of addiction liability" of each drug. In this ratio the minimum therapeutic dose in man does not necessarily imply the same therapeutic effect. We are relating addictive potency to the doses actually used in clinical practice. (The calculated index for each drug is shown in Table I.) The higher the value of this index then the greater should be the danger of causing addiction in man but a glance at the relative sizes of the indices shows that they do not always reflect clinical experience in man.

The indices would suggest that ketobemidone should be more addicting than heroine, that dihydromorphinone and alphaprodine should be more addicting than morphine, and that levorphan and meperidine should be less addicting than codeine. These do not correspond with the results of clinical trials (Isbell (7)).

The lack of precise correlation between the two sets of potencies (for production of excitation in the cat and addiction liability in man) could be due to the difficulties of comparing the relative addiction liabilities in man. However, it would appear that the relative size of the dose required to produce excitation in the cat gives a better prediction of addiction liability than does the magnitude of Mayor's index.

Not all the drugs which were found to cause excitation have been reported to produce addiction in man. We can find no evidence stating that apomorphine, apocodeine, or pholcodine cause addiction. It may be that the methods of using these drugs do not favor the development of addiction.

None of the drugs which did not cause excitation can be considered to show marked analgesic properties but it is doubtful whether there is a relationship between the intensity of the exciting effect of a compound and its analgesic potency. Some of the exciting drugs are only weak analgesics.

Although a precise correlation between the ability to produce excitation in the cat and the danger of producing addiction in man has not been found for the group of drugs studied, the relation is sufficiently close to suggest that

the cat injection test could be used to indicate the danger of inducing addiction by this class of drugs.

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THE HEXOSAMINE AND HYDROXYPROLINE CONTENTS OF THE OVIDUCTS OF THE ESTROGENIZED IMMATURE PULLET AND OF THE NORMAL LAYING HEN[1]

P. A. ANASTASSIADIS

Abstract

Estradiol benzoate, 1 mg per day, plus testosterone propionate, 1 mg per day, were administered to sexually immature pullets for 10 days. The dry matter, hexosamine, and hydroxyproline contents of the hypertrophied oviducts and of their major anatomical parts were in general similar to the corresponding data obtained for the oviducts of mature laying hens. Additional treatment with progesterone, 0.5 mg per day, did not affect in any definite way the weight and dry matter, hexosamine, or hydroxyproline contents of the hypertrophied oviducts of the immature pullets. Additional treatment with progesterone, 1.0 mg per day, significantly depressed the weight and dry matter contents of the hypertrophied oviducts and of their major anatomical parts, except for the vagina.

Introduction

Estrogen-induced hypertrophy of the oviducts of the immature pullet may be modified by concurrent administration of progesterone (PGST). The effect will vary with the levels and ratios of estrogen and PGST. If the levels of estrogen and PGST are relatively high, then PGST will depress the estrogen-induced hypertrophy (1, 2, 3). If the level of estrogen is fairly high, then small doses of PGST will enhance the estrogen-induced hypertrophy (4). Yet again, larger doses of PGST will enhance the effect of small doses of estrogen (5).

Progesterone has qualitative effects on the response of oviduct to estrogen as well as quantitative effects. Hertz, Fraps, and Sebrell (6) showed that estrogen by itself did not induce avidin formation in the oviduct whereas estrogen combined with progesterone did. Brant and Nalbandov (7) have drawn attention to the role of progesterone in causing accumulation of albumen in the glands of the magnum and even in causing secretion of the albumen into lumen of the oviduct.

Testosterone may enhance estrogen-induced hypertrophy of the pullet's oviduct (8). Both the weight and hexosamine content of the magnum of estrogenized immature pullets have been found to display a greater relative increase with additional androgen treatment than did the corresponding values for the shell gland and vagina (9). The hexosamine and hydroxyproline contents of a tissue may afford valuable information as to the state of the intercellular material of the tissue. The hexosamine content reflects, even if only approximately, the amount of interfibrillar intercellular material, while the hydroxyproline content reflects, with a better approximation, the amount of collagenous fibrillar intercellular material (9). This fundamental concept is shared by many workers who now express the fibrillar density of a tissue by

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means of ratios based on the hydroxyproline and hexosamine contents of the tissue, e.g. the hexosamine/collagen ratio (10).

The observations mentioned above have now been extended to a study of the effects of progesterone on the weight and dry matter, hexosamine, and hydroxyproline contents of the oviducts of sexually immature pullets treated with estradiol benzoate (ODB) plus testosterone propionate (TST).

The present study was undertaken in an effort to explore the possibility that ODB + TST, or ODB + TST + PGST, might give rise to an oviduct closer in composition to that of the normal laying hen than would ODB alone.

Experimental

General Experimental Methods

Twenty-four crossbred (New Hampshire × Barred Plymouth Rock) pullets, aged 8 weeks, were assigned at random among four groups and housed in individual cages. The four groups were given the following hormonal treatments by intramuscular injection in sesame oil solution:

Group I: sesame oil diluent only,
Group II: 1.0 mg ODB + 1.0 mg TST per day,
Group III: 1.0 mg ODB + 1.0 mg TST + 0.5 mg PGST per day,
Group IV: 1.0 mg ODB + 1.0 mg TST + 1.0 mg PGST per day.

The ODB was given as 'Progynon B' (Schering), the TST as 'Oreton' (Schering), and the PGST as 'Proluton' (Schering). The injections were given for 10 days. On the morning after the last injection, the pullets were killed by decapitation and bled. The livers, kidneys, ovaries, and oviducts were removed and weighed. The magnum, isthmus, shell gland, and vagina of each oviduct were then separately weighed and analyzed for dry matter and total hexosamine and hydroxyproline. The birds were fed individually so that each bird consumed the same total quantity of food during the experiment.

Analytical Methods

The tissue samples were freeze-dried and stored in the freezer pending analysis. Before analysis, the samples were equilibrated with laboratory air and then subsampled for analysis.

Dry matter was determined by drying the tissues to constant weight at 100° C in an air oven. Hexosamine and hydroxyproline were determined as described elsewhere (9).

Experimental Results and Discussion

The average results for dry weight, liver weight, kidney weight, ovary weight, and oviduct weight are presented in Table I. None of the differences in live weight attained significance at $P = 0.05$. ODB + TST produced a highly significant increase ($P < 0.01$) in liver weight similar to that observed previously for similar treatments (11). PGST did not modify this response significantly.

None of the differences in kidney weight attained significance at $P = 0.05$. This is understandable in so far as estrogen by itself increases kidney weight (3)

TABLE I

Average results for live weight and weights of liver, kidney, ovary, and oviducts. Six pullets per group. Duration of experiment 10 days

	Group			
	I	II	III	IV
Estradiol benzoate, mg per day	Nil	1.0	1.0	1.0
Testosterone propionate, mg per day	Nil	1.0	1.0	1.0
Progesterone, mg per day	Nil	Nil	0.5	1.0
Live weight, initial, kg*	0.92±0.054	0.92±0.032	0.94±0.032	0.91±0.062
Live weight, final, kg*	1.14±0.044	1.16±0.047	1.22±0.034	1.18±0.065
$\frac{\text{Live weight final}}{\text{Live weight initial}} \times 100$	125 ±2.76	126 ±3.76	130 ±3.47	130 ±6.60
Liver, g	21.1±1.16	31.5±1.81	35.1±1.59	32.4±2.02
Kidney, g	7.2±0.32	7.9±0.48	8.5±0.56	8.8±0.66
Ovary, mg	307 ±27	257±23	244 ±19	289 ±24
Whole oviduct, g	0.14±0.02	13.1±0.98	9.2±0.49	6.6±0.23
Magnum, g	—	4.6±0.23	3.2±0.20	1.6±0.16
Isthmus, g	—	0.9±0.10	0.8±0.07	0.4±0.03
Shell gland, g	—	5.1±0.54	3.5±0.18	2.9±0.10
Vagina, g	—	1.9±0.25	1.2±0.12	1.1±0.14

*Initial weighings were made 15 days before slaughter.
NOTE: Each average is accompanied by its standard deviation.

whereas TST by itself depresses kidney weight (12). The tendency of PGST to increase the weight of the kidney of the hormonally treated birds, though noteworthy, did not attain significance at $P = 0.05$.

None of the treatments affected ovary weight significantly.

The results for oviduct weight show that the single dose of PGST depressed the response of the weight of the oviduct and of its parts to treatment with ODB + TST to a highly significant degree, and that the depression was greater with the double dose of PGST. These results are in general agreement with the results previously reported for comparable treatments (3) so far as total oviduct weight is concerned.

The foregoing results for live weight and weights of liver, kidney, ovary, and total oviduct showed that the responses of the pullet to the hormonal treatment were in accord with previous observations (3, 11).

TABLE II

Average percentage of dry matter of oviducts and their parts. Six pullets per group

	Group			
	I	II	III	IV
Estradiol benzoate, mg per day	Nil	1.0	1.0	1.0
Testosterone propionate, mg per day	Nil	1.0	1.0	1.0
Progesterone, mg per day	Nil	Nil	0.5	1.0
Total oviduct	19.4±0.77	19.0±0.26	19.3±0.35	15.6±1.12
Magnum	—	25.4±0.40	26.7±0.57	21.2±1.81
Isthmus	—	19.6±0.20	20.4±0.25	19.4±0.84
Shell gland	—	14.5±0.29	13.9±0.24	12.6±0.88
Vagina	—	14.4±0.29	14.3±0.31	13.4±0.66

NOTE: Each average is accompanied by its standard deviation.

The average results for the dry matter content of the oviduct tissues, are presented in Table II. Statistical evaluation of these data was complicated by the fact that the variances of the groups were substantially different. Welsh (13) and Asprin (14) have elaborated a solution for this problem. This solution is based on the calculation that the fraction

$$d/\sqrt{s_{\bar{x}_1}^2 + s_{\bar{x}_2}^2},\ \text{where } s_{\bar{x}_1}^2 = s_1^2/n_1 \text{ and } s_{\bar{x}_2}^2 = s_2^2/n_2$$

has a "t" distribution with ν degrees of freedom, where ν is given by

$$\frac{1}{\nu} = \frac{1}{n_1 - 1}\left(\frac{s_{\bar{x}_1}^2}{s_{\bar{x}_1}^2 + s_{\bar{x}_2}^2}\right)^2 + \frac{1}{n_2 - 1}\left(\frac{s_{\bar{x}_2}^2}{s_{\bar{x}_1}^2 + s_{\bar{x}_2}^2}\right)^2.$$

Hence when $n_1 = n_2 = n$ we have

$$\frac{1}{\nu} = \frac{s_{\bar{x}_1}^4 + s_{\bar{x}_2}^4}{(n-1)(s_{\bar{x}_1}^2 + s_{\bar{x}_2}^2)^2}.$$

It is advisable to use this test of significance whenever there is no sound basis for the assumption that the statistics to be compared have equal variances.

Application of this statistical test (see Table III) showed that the larger level of PGST had increased the moisture content of the tissues of the oviduct,

TABLE III

Statistical analysis of data for dry matter contents of oviducts from Table II

Comparison	Ratio second to first	D.F.	t	P
Total oviduct				
I vs. IV	0.81	8.83	2.72	<0.025
II vs. IV	0.82	5.53	3.22	<0.025
Magnum				
II vs. IV	0.83	5.50	2.30	<0.100
Shell gland				
II vs. IV	0.87	6.08	2.02	<0.100
Vagina				
II vs. IV	0.93	6.77	1.42	<0.250

whereas the lower level had no such effect. The depressant effect of larger doses of PGST on the oviduct, therefore, was not primarily a dehydration.

The results for the hexosamine and hydroxyproline contents of the oviducts and their component parts are presented in Table IV. The values relate to composite samples and the standard deviations relate, therefore, to the means of the chemical analyses and not to the biological means.

The hexosamine contents of magnum, shell gland, and vagina of Group II were similar, though slightly higher, than the values previously reported for birds treated with ODB + TST (9). In addition, the isthmus was found to have a hexosamine content approaching that of the vagina. The highest

TABLE IV

Hexosamine and hydroxyproline contents of immature pullets treated with ODB + TST and with ODB + TST + PGST. Results on pooled samples from each group of six birds

			Hexosamine		Hydroxyproline	
Group	Treatment	Part of oviduct	Mg per g dry tissue	Total mg	Mg per g dry tissue	Total mg
I	Nil	Whole oviduct	6.4±0.28	0.17	20.1	0.54
II	ODB + TST	Magnum	20.7±1.11	23.9	2.8	3.24
		Isthmus	20.1±0.59	3.5	6.3	1.08
		Shell gland	8.8±0.30	6.5	8.7	6.4
		Vagina	9.4±0.60	2.5	14.5	3.9
		Calculated for whole oviduct less infundibulum	15.6	36.4	6.3	14.6
III	ODB + TST + PGST (single dose)	Magnum	23.2±0.41	20.0	2.6	2.2
		Isthmus	18.8±0.59	2.9	6.0	0.9
		Shell gland	8.5±0.21	4.1	6.9	3.4
		Vagina	10.0±0.76	1.7	14.9	2.5
		Calculated for whole oviduct less infundibulum	17.2	28.6	5.4	9.0
IV	ODB + TST + PGST (double dose)	Magnum	21.2±0.91	7.1	4.6	1.6
		Isthmus	14.9±0.68	1.2	8.7	0.7
		Shell gland	7.1±0.57	2.6	10.7	3.8
		Vagina	10.2±0.67	1.5	17.0	2.5
		Calculated for whole oviduct less infundibulum	13.4	12.3	9.3	8.6

NOTE: The analyses were made on pooled samples and the standard deviations of the means relate to the variation of the chemical analyses.

hydroxyproline content was in the vagina; this, doubtless, is an expression of its relatively high collagen content. The data do not reveal any definite effect of PGST on the hormonally hypertrophied oviduct's content of hexosamine, but it may be noted that the hydroxyproline contents of Group IV were increased. This suggests that the depressant effect of PGST on the oviduct hypertrophy fell relatively more heavily on non-collagen constituents.

Neither the foregoing data, nor those reported previously, include values for the oviduct of the normal mature laying hen which might serve as a basis for comparison. Accordingly, the oviducts were removed from three laying hens and the magnums, shell glands, and vaginas were dissected, pooled, and analyzed for dry matter, hexosamine, and hydroxyproline. The results are presented in Table V. In Table V, values for oviducts hypertrophied by treatment with exogenous hormones are also reported. These values are the unweighted arithmetical averages of the values reported above and previously (9). As regards dry matter content, it can scarcely be said that one of the artificially hypertrophied oviducts resembles the normal oviduct more than another. As regards hexosamine content, there are indications that inclusion of TST and/or PGST along with ODB may give an oviduct closer in composition to the normal mature oviduct than does ODB alone. As regards hydroxyproline, the shell gland and vagina of the normal oviduct had distinctly higher contents than those of any of the artificially hypertrophied oviducts, but this may be related to an increasing content of collagen with age. It must be remembered, furthermore, that the oviduct of the laying hen is subject to cyclical changes in weight during egg formation, these changes being most pronounced in the magnum (15). Such changes are not likely to complicate observations on the artificially hypertrophied oviduct.

TABLE V

Comparison of average dry matter, hexosamine, and hydroxyproline contents of oviducts of hormonally treated immature pullets and of mature laying hens

Part of oviduct	Immature pullets				Mature laying hens
	Nil*	ODB	ODB + TST	ODB + TST + PGST	
		Dry matter, %			
Magnum	(19.1)	22.3	25.8	24.0	26.3
Shell gland		15.4	15.0	13.3	13.9
Vagina		15.5	14.9	13.9	17.1
		Hexosamine, mg per g dry tissue			
Magnum	(6.4)	13.8	19.8	22.2	17.1
Shell gland		5.5	7.8	7.8	6.4
Vagina		6.6	9.1	10.1	9.1
		Hydroxyproline, mg per g dry tissue			
Magnum	(16.9)	2.9	2.3	3.6	2.9
Shell gland		5.7	7.2	8.8	10.8
Vagina		10.5	12.4	16.0	18.9

*The data for the rudimentary, unhypertrophied oviducts of Group I are not strictly comparable with those for the other groups, and relate to the entire organ.

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NOTES

THE REDUCTION OF ADRENOCHROME WITH THIOLS

R. A. Heacock and Barbara D. Scott[1]

The formation of a "leuco" derivative of adrenochrome by the action of hydrogen sulphide was first reported in 1937 (1), and since then several other compounds containing the —SH group have been shown to decolorize adrenochrome solutions, i.e. B.A.L. (2,3-dimercapto-1-propanol) (2), thioglycollic acid (3, 4), gluthathione (4), and cysteine (4, 5). In view of the possible physiological implications of these reactions, and the somewhat limited nature of previous studies, the products obtained by the action of the above-mentioned reducing agents on adrenochrome, together with those produced by penicillamine (β,β-dimethylcysteine) and ergothioneine, have been subjected to paper chromatographic analysis. A technique similar to that previously used by the authors to study the action of a number of other reducing agents on adrenochrome (4, 5) was employed.

Hydrogen sulphide, B.A.L., and thioglycollic acid caused almost instantaneous decolorization of adrenochrome solutions, but the four amino acids were considerably slower in their action and several minutes were usually required to complete the reaction. Spots, consisting of 25–35 μl of the reaction mixture (prepared from adrenochrome (0.02 g) in water (2.0 ml), treated with a slight excess of reducing agent at room temperature until the red color of the solution was discharged, and filtering if necessary to obtain a clear solution) were examined on Whatman No. 1 paper (previously washed for 48 hours with 2% acetic acid) using 2% acetic acid as the running solvent. After being dried, the chromatograms were sprayed with: (*a*) Ehrlich's reagent; (*b*) cinnamaldehyde; (*c*) *p*-dimethylaminocinnamaldehyde (6) (DMCA); (*d*) Gibb's reagent; and (*e*) Folin and Ciocalteu's reagent. The R_f values of the main products and the colors obtained with Ehrlich's reagent are shown in Table I. 5,6-Dihydroxy-*N*-methylindole (I), which has been isolated in some instances from the reduction products of adrenochrome (e.g. with sodium hydrosulphite (7), hydrogen (catalytic) (7), ascorbic acid (5), and sodium borohydride (5)) has an R_f of ca. 0.40 to 0.46 under the conditions used in this investigation (4, 5). Thus it would appear that all the thiols mentioned in Table I gave (I). In all the cases studied, other as yet unidentified spots were observed on the chromatograms which gave indole chromogenic reactions. In five out of these six cases a relatively weak spot giving a violet color with Ehrlich's reagent was observed with an R_f of ca. 0.70. (An Ehrlich positive spot has previously been observed in this R_f zone with several other reducing agents (4).) Thioglycollic acid appears to give, together with (I) and two minor products with R_f's of 0.16 and 0.71, a substance (II) with an R_f only slightly higher than (I); the

[1]Née Laidlaw.

TABLE I

Paper chromatography of adrenochrome reduction products

Reducing agent	Average R_f values of major spots and color with Ehrlich's reagent
1. Hydrogen sulphide	0.46(b.v.); 0.70(v)^{w}
2. B.A.L.	0.46(b.v.); 0.70(v)^{w}; 0.78(o.p.)^{*s}
3. Thioglycollic acid	0.16(b.v.); 0.46(b.v.); 0.51(b.v.); 0.71(v)^{w}; 0.82(y)^{*s}
4. Penicillamine	0.05(b.v.)^{w}; 0.43(b.v.); 0.68(v); 0.84(y)^{*s}
5. Glutathione	0.44(b.v.); 0.61(b.v.); 0.75(g.b.)^{*s}
6. Cysteine	0.12(y.g.); 0.27(b.g.); 0.45(b.v.)^{w}; 0.50(b.v.); 0.70(v); 0.84(y.g.)^{*s}

ABBREVIATIONS: b.v. = blue-violet; v = violet; o.p. = orange-pink; y = yellow; g.b. = green-brown; y.g. = yellow-green; b.g. = blue-green; w = only a relatively weak reaction observed; s = color develops slowly; * = these spots are probably due to excess reducing agent.

NOTE: When the chromatograms were sprayed with Gibb's reagent, brown spots were observed where a positive reaction was obtained with Ehrlich's reagent; similarly Folin and Ciocalteu's reagent gave blue-grey spots. DMCA gave dark bluish-green colors with the indole derivatives; the spots due to excess reducing agents were observed as colorless zones on the paper, which assumed a general pink color (after about 12 hours).

two products (I) and (II) were not efficiently separated with the solvent system in use. However, after treatment of the reaction mixture with a slight excess of solid sodium bicarbonate, (I) could be totally extracted with peroxide-free ether whilst (II) remained in the aqueous phase. (It was shown in a similar experiment, using ascorbic acid as the reducing agent, that (I) was completely extracted into the ether phase after similar treatment of the reaction mixture.) There was only a slight difference in shade in the colors given by (I) and (II) with Ehrlich's reagent, but cinnamaldehyde gave an orange color with (I) and a violet-brown color with (II) and DMCA gave a blue-green color with (I) and a blue color with (II). The structure of (II) has not yet been determined; however it appears to contain a carboxylic acid group and an indole nucleus. Ergothioneine does not give (I), but a spot giving indole color reactions was observed at R_f 0.75 ± 0.05. A different mode of action in this case would not be altogether unexpected, since the —SH group in ergothioneine is quite different in character from that in the other thiols studied (cf. Bell (8)). 2-Iodo- and 2-bromo-adrenochrome are also rapidly reduced by hydrogen sulphide and B.A.L. to mixtures of indoles including the corresponding 2-halogeno-5,6-dihydroxy-*N*-methylindoles.

These investigations are being continued and further details will be published elsewhere. These studies were carried out under the auspices of the Saskatchewan Committee on Schizophrenia Research and supported by the Saskatchewan Government, the Department of National Health and Welfare, and the Rockefeller Foundation.

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Symposium on Metabolism of Insecticides

This symposium, sponsored jointly by the Agricultural Chemistry Subject Division of the Chemical Institute of Canada and the Entomological Society of Canada, was held at Guelph on October 31 and November 1, 1958. Dr. E. Y. Spencer of the Science Service Laboratory at the University of Western Ontario was Chairman of the organizing committee and editor of the symposium, as well as Chairman of the Friday sessions. Dr. H. Martin, Director of the Science Service Laboratory, London, was Chairman of the Saturday sessions.

INTRODUCTION

E. Y. Spencer

The great increase recently in the use of organic chemicals in the control of undesirable insects has brought many problems. Among them are the development of resistance, greater specificity of action and metabolism of the materials. To attempt to solve or at least examine these problems has required the participation of both entomologists and chemists. Often the mammalian physiologist or biochemist is working independently of the insect physiologist or toxicologist. It is therefore of advantage to bring these groups together by participation in a symposium such as is sponsored for the first time jointly by the Chemical Institute and the Entomological Society of Canada. It is hoped that as a result of such a conference the pressing problems of resistance, greater specificity of action, potentiation, and even mode of action may be clarified to some extent.

INSECTICIDE RESISTANCE AS A WORLD PROBLEM[1]

A. W. A. Brown

Many Canadians have the impression that insecticide resistance is essentially an American product. There are confirmed cases of the development of resistance in 72 species of arthropods, and no less than 50 of them occurred in the U. S. A. (1). The parathion resistance of fruit-tree red spider in British Columbia (2) was foreshadowed by its previous occurrence in the state of Washington (3), and that of the two-spotted mite in Ontario greenhouses (4) followed its general prevalence in the northeastern U. S. A. (5). In southern Ontario the DDT resistance of the cabbage looper (6) and the DDD resistance of the red-banded leaf-roller (7) have their antecedents in New York State (8, 9); and the aldrin resistance of the onion maggot in southwestern Ontario and British Columbia (10) follows the discovery of dieldrin resistance in this species in Wisconsin, Washington, and Oregon (11, 12).

But many resistance problems range widely over the world. DDT resistance in the codling moth has developed in South Africa (13) and South Australia (14) as well as in the U. S. A. (15) and Canada (16). Strains of citrus thrips that resisted tartar emetic appeared in the Transvaal (17) as well as California (18). Parathion-resistant red spiders on apple have recently been reported from Holland (19) and South Africa (20), and DDT-resistant cabbage worms from New York and Wisconsin (8, 21) and from Japan (22). DDT-resistant bedbugs are known not only in American cities but also in the Mediterranean, the Caribbean, and the Far East (23). Strains of the cat flea resistant to chlorinated hydrocarbons are no longer exclusive to the U. S., but also occur in equatorial South America and Hong Kong. The mosquito *Culex fatigans* has developed a high resistance to chlorinated hydrocarbons in at least 12 countries besides the state of California.

The development of resistant populations of the housefly has been recorded in virtually every country of the world except mainland China (23). The cattle tick in Australia and the blue tick in South Africa have shown a remarkable parallelism despite being separated by 5000 miles of ocean. The former, *Boophilus microplus*, was recognized as arsenic resistant in 1937, BHC resistant in 1952, and DDT resistant in 1955 (24, 25); while the latter, *B. decoloratus*, was found arsenic resistant in 1937, BHC resistant in 1948, and DDT resistant in 1956 (26, 27).

Origins and Mechanisms

The fundamentals of the resistance phenomenon are world-wide. That it is due to a process of Darwinian selection has been proved by the experience in London, Palermo, and Orlando (28, 29, 30) that dosages which do not kill do

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not increase resistance, and by experiments in Berlin and Belleville (31, 32) demonstrating that the insecticides are not themselves mutagenic; proof that resistance pre-exists in a small proportion of the natural population even before the insecticide is introduced has been obtained in Taiwan, Northern Nigeria, and French West Africa (33, 34, 35). In fact, insecticide resistance has provided the best example of the essential truth of neo-Darwinism, and a fitting tribute for this centennial year of the Darwin–Wallace theory.

The discovery that there are essentially two types of resistances within the chlorinated hydrocarbons, one to DDT and analogues, and the other to cyclodiene derivatives (dieldrin, heptachlor, etc.) and gamma-BHC, was made by Busvine (36) from his studies of the housefly. The distinctness of these two entities has been subsequently confirmed in many species of mosquitoes, two species of bedbugs, and two of fleas, in the body louse and the German roach, and in the two species of cattle tick mentioned above. This distinctness will probably be found to hold good in resistant strains of agricultural insects also.

Researchers in Copenhagen, London, and Rome have shown that the genetical basis of chlordane–BHC resistance in the housefly is different from that of DDT resistance (37, 38, 23). That the genetic origin of DDT resistance is essentially monofactorial has been demonstrated for Illinois houseflies by Lichtwardt (39) and for Italian houseflies by Milani (40). The evidence so far indicates that the same gene allele is involved in both localities. The DDT resistance of *Aedes aegypti* in Trinidad and of *Anopheles sundaicus* in Java is probably due to a single gene (41, 42). The dieldrin resistance of *Anopheles gambiae* in Northern Nigeria, which extends to BHC but not to DDT, has been proved to be monofactorial (43); while the chlordane resistance of the German cockroach in the U. S. A., extending to dieldrin but not to DDT, is due to several genetic factors (44), as in the housefly. On the other hand, a single gene in *Drosophila melanogaster*, located at map distance 66 on chromosome II, has been found by Japanese workers to impart a resistance not only to DDT but also to BHC and many other insecticides (45, 46). It is this type of non-specific tolerance which, if imparted by a number of genes of minor effect, would constitute the "vigor tolerance" recently characterized by Hoskins and Gordon (47).

The biochemical mechanism of specific DDT resistance in the housefly has been demonstrated by many workers, notably Kearns and his associates, to be one of detoxication by a dehydrochlorinating enzyme (48, 49). Production of the non-toxic metabolite DDE has been demonstrated in DDT-resistant *Aedes aegypti*, *Culex fatigans*, and *Anopheles sundaicus* (50, 51, 52), but not in resistant body lice (53). Resistance to gamma-BHC has been shown to derive partly from detoxication and partly from reduced absorption (54, 55, 56). Although notable research on dieldrin resistance in the housefly has been recently performed at Riverside, Savannah, Urbana, and Slough, the researchers themselves are the first to admit that no mechanism has yet been discovered which could account for this intense type of resistance (57).

The Resistance Problem

Insecticide resistance, then, is a problem of universal application and involves workers in many lands. The bulk of our fundamental knowledge of the phenomenon derives from insects of public-health rather than agricultural importance. This, of course, is partly due to their being easier to rear in the laboratory. But it is also due to the world-wide importance of DDT and other chlorinated hydrocarbons as weapons to attack the insect vectors of disease. Vast sums of money are at stake in international undertakings to eradicate the transmission of malaria in Central and South America, the bulk of southern Asia, and many parts of Africa (58). The DDT resistance of *Anopheles sundaicus* in northern Java and of *A. stephensi* in the Persian Gulf region offer a serious threat to the success of these undertakings, as also does the dieldrin resistance of *A. gambiae* in Northern Nigeria, Upper Volta, and Liberia and of *A. albimanus* in Central America (23). The value of DDT louse powder as a safeguard against typhus has been cancelled by resistance in the Far East and the Palestine area, as well as in certain spots in Africa. Resistance quickly negates the effectiveness of chlorinated hydrocarbons against *Culex fatigans* in filariasis control, while the program of eradication of *Aedes aegypti*, the vector of yellow fever, has encountered strong DDT resistance in Trinidad, Venezuela, and other parts of the Caribbean area (23).

It was these considerations that made international co-operation on the resistance problem absolutely essential, and in 1956 the national scientists concerned entrusted the World Health Organization with the task of mobilizing it. Very quickly liaison was established with over 300 workers throughout the world, and was maintained by the regular distribution of information circulars on the subject, by personal visits and correspondence, by sending outstanding research workers to various parts of the world, and by holding large regional seminars on the resistance problem. New research projects were opened up and supported either directly by WHO or by national or independent granting agencies. The result has been not so much the accretion of new knowledge as the promulgation of existing knowledge, so that all those involved in the technical direction of insect control are well briefed on resistance and know the colleagues they can turn to in any part of the world.

Standard Test Methods

One of the first practical necessities of the program was to devise a set of standard tests for resistance which could be simply applied in the field. Test methods and kits for adult mosquitoes, mosquito larvae, and body lice were developed by WHO and are now in use (59, 60). They enable the population to be assessed for its level of susceptibility or resistance by obtaining a dosage–mortality relationship from which the LC_{50} may be derived for the insecticide concerned. The U. S. Armed Forces have made available certain test methods, originally devised by the U.S.D.A. laboratory at Orlando, whereby the LT_{50} may be obtained for samples of German roaches and bedbugs, and have promoted a rough test for resistance in houseflies (61). These standard

procedures not only constitute a tool to detect resistance, but also provide the means whereby suspected resistance may be proved or disproved and thus quickly removed from the level of mere rumor. The rapid promulgation of valid test findings from the field is one of the aims of the WHO program.

The test method for adult mosquitoes, which was the most difficult to develop, is based upon specially prepared papers impregnated with graduated doses of the insecticide dissolved in mineral oil (62). This principle is also applicable to adult *Phlebotomus* and *Drosophila*, and possibly other Diptera, fleas, bedbugs, and ticks. It would appear likely that the present delay in confirmation or refutation of suspected resistance in agricultural insects might be eliminated by devising standard field tests of this nature.

Substitute Insecticides

A periodic check on the susceptibility levels enables the operator to be forewarned should resistance arise, and he should then look for substitute insecticides. For example, the DDT-resistant *Anopheles sundaicus* in Java are now being satisfactorily controlled by dieldrin, and the DDT-resistant *Aedes aegypti* in Trinidad by BHC. The usual progression is from DDT through DDT resistance to BHC or dieldrin. Many populations of body lice, bedbugs, and fleas have reached this stage, but growing BHC or dieldrin-resistance makes it mandatory to hold in readiness an organophosphorus compound such as malathion or diazinon. The cattle tick and blue tick have passed this stage, and already diazinon dips are in use in South Africa. The German roach has graduated directly from chlordane through chlordane resistance to malathion, and now malathion-resistant populations have appeared in Alabama and Florida. Larvae of *Culex tarsalis* and *Aedes nigromaculis* mosquitoes in California became so resistant to chlorinated hydrocarbons that parathion, EPN, and malathion were substituted; but malathion resistance of *Culex* has appeared in Fresno county and parathion resistance of *Aedes* in Kings county (63, 64). The housefly graduated to organophosphorus compounds around 1953, and now diazinon resistance has developed in Denmark, Switzerland, Italy, and New Jersey (65, 23, 66, 67), and malathion resistance in Florida, Georgia, and Arizona (68, 69). This is just about the end of the road, especially since pyrethrin resistance is now known from Sweden (70) and increased pyrethrin tolerance is general in the U. S. A. (71).

There is some small degree of comfort in the cross-resistance pattern to the different organophosphorus compounds. Diazinon-resistant strains of houseflies from Denmark and Italy are still susceptible to malathion, while a malathion-resistant strain from Florida was comparatively susceptible to diazinon (72). OP-resistant flies remain reasonably susceptible to Dipterex (73). As for the physiological basis of OP resistance, it has been found that the malathion-resistant strain from Savannah withstands malathion poisoning by retaining more cholinesterase (74) and accumulating less acetylcholine than the normal (75). It has been reported that parathion-resistant flies degrade paraoxon more rapidly than normal (76). Unfortunately, selection pressure

from organophosphorus compounds such as parathion, diazinon, and malathion greatly increases the resistance to chlorinated hydrocarbons (77, 78).

Although the co-operative international program has witnessed considerable advances in the understanding of resistance, it has not produced any striking countermeasures. However, much can be done in the light of knowledge and common sense. In certain instances, as in *Anopheles gambiae*, it is possible to test in the field for the resistance potential of the mosquito population to a possible insecticide such as dieldrin; where the gene for dieldrin resistance is present one can avoid flying in the face of certain trouble. The test kits carry not only DDT but also BHC and/or dieldrin, so that cross-resistances may be checked and the validity of a substitute chlorinated hydrocarbon established. Steps are being taken to have certain organophosphorus compounds ready for emergencies, such as malathion louse powders cleared for toxic hazard, or wettable powders of diazinon to which have been added certain extenders, such as DDT or chlorobenzilate, to prolong its residual activity against mosquitoes. Residual deposits of diazinon can eliminate DDT-resistant *Anopheles subpictus* in India (79), and malathion residuals have proved much more effective against resistant *A. albimanus* in Central America than the initial experiments against resistant *A. sacharovi* in Greece had indicated (80). For housefly control, dry baits containing Dipterex succeed against populations resistant to residual sprays of malathion or diazinon.

Possible Ultimate Solution

The international program has, however, turned up the remote possibility of a single answer to the resistance problem. This lies in the alternation of two insecticides negatively correlated to each other with respect to cross-resistance. For example, a certain organophosphorus preparation produced by the U. S. Department of Agriculture at Beltsville, and called diisopropyl tetrachloroethylphosphate (81), proved to be more toxic to DDT-resistant houseflies than to DDT-susceptible ones; that is, the cross-resistance to this material and to DDT was negatively correlated. When a DDT-resistant strain containing a few susceptible individuals was submitted to selection pressure at partial kill by this compound, it returned to a DDT-susceptible strain in three generations. Unfortunately, attempts to resynthesize a preparation of this organophosphorus compound with these peculiar insecticidal properties have been unsuccessful. Another negatively correlated compound is cetyl bromoacetate, found by Ascher in Rome to kill resistant housefly strains faster than normal strains (82, 83). Field experiments are now being completed in Denmark to ascertain whether the insecticidal action of cetyl bromoacetate can reverse the resistance levels of isolated farm populations. The results of concomitant laboratory studies, which are now available, are, however, not promising. It may be that the genetic differences between Ascher's strains and the Danish populations cancel this effect.

The most promising lead, at least in theory, comes from Japan. At Osaka University it has been found by Ogita (84, 85) that strains of *Drosophila*

carrying the gene allele for resistance to DDT and BHC are more susceptible than normal to phenylthiourea (PTU, misnamed phenylthiocarbamide or PTC by the geneticists). Moreover the PTU susceptibility is due to the same gene allele as the DDT resistance. So the negative correlation of these two insecticidal compounds is genetically fixed. The results of the transference of this study from *Drosophila* to the housefly are now awaited.

During the coming years, the tolerance levels of most populations under insecticide pressure will continue to rise, and ever more species will acquire resistant strains. Our first task is the doleful one of ascertaining these occurrences and putting them accurately on record. The next chore is to determine their cross-resistance patterns, in order to ascertain alternative insecticides that are still effective. This may be followed by fundamental studies of the physiological mechanism and genetic origin of the resistance. The genetic studies would provide the means of testing still-susceptible field populations for their genic potential for developing resistance. The physiological and biochemical studies might result in the discovery of compounds to which resistance will not develop (perhaps chloroacetate esters and certain inorganic insecticides fall in this class), or which are negatively correlated to the existing insecticides and thus can reverse the resistance to them. The further alternative of abandoning the use of insecticides is an unlikely one since it appears that insecticides are here to stay. Therefore, the search for remedial insecticides should be pushed persistently against all disappointments, not only by academic and governmental researchers but also by industry, so that we may achieve the answers which the resistance problem demands.

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MECHANISMS OF RESISTANCE TO CHLORINATED HYDROCARBONS[1]

HERBERT H. MOOREFIELD

Insect resistance to insecticides is as old as our concept of control by chemicals and the concern over this phenomenon has been ever increasing since the advent of widespread use of synthetic chemicals for pest control. Obvious implications of resistance have stirred the interest of many workers in widely different disciplines and the literature is now replete with partial explanations for these conditions.

Through the co-ordinated efforts of such groups as the World Health Organization, the concepts behind insect resistance are becoming better focused, and centrally cumulated information is being disseminated to all investigators interested in these problems. Some of the basic mechanisms implicated in resistance to the chlorinated hydrocarbon insecticides that are currently receiving attention are:

1. Reduced uptake or absorption of the insecticide by tolerant species.
2. The ability to store the toxicant in fat depots separated away from the site of action.
3. Changes in the nature of nerve sheaths or other target sites.
4. Metabolism, enhanced detoxication, or increased excretion of the insecticide.
5. Changes in behavioristic patterns or avoidance of residue surfaces.

These topics have all been excellently reviewed recently (1, 2, 3, 4) and the status of metabolism in resistance to the chlorinated hydrocarbon insecticides will be examined in this paper.

Our knowledge of metabolism of insecticides by insects has been principally gained through researches designed to learn more about resistance, through comparative mammalian detoxication studies, and through experiments executed to disclose the mode of action of these chemicals. Such information can, in turn, be utilized to reduce the potential health hazards encountered in the use of toxic compounds and to produce highly effective control of pest species with more efficient molecules.

Unfortunately, at the present time, it is possible in only a very few instances to interpret the mechanism of action of insecticides in strictly biochemical terms. Understanding of the many-faceted resistance problem is equally meager, but the metabolic role is certainly the best documented phase as of this date. Selective toxicity in which a compound is specifically lethal to certain insects and harmless to others, or relatively safe to mammals and deadly to insects, can frequently be traced to differences in metabolism of the insecticide.

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Contribution from the Boyce Thompson Institute for Plant Research, Yonkers, New York. This paper was presented at the Symposium on Metabolism of Insecticides held at the Ontario Agricultural College, Guelph, Ontario, October 31 and November 1, 1958.

The metabolic fate of an insecticide will be determined by its inherent chemical nature as well as by the metabolic capacities of the tissues it encounters.

The toxicity of an insecticide may be manifested directly, or may reflect the potency of a metabolic product derived from the original compound. Thus, an insect may be killed as a result of its tissue components being incapable of metabolizing the insecticide or because of the accumulation of a poisonous metabolite.

Classification of acquired housefly resistance to the chlorinated hydrocarbons conveniently falls into two distinct groups. The first group contains DDT, methoxychlor, and other structurally related compounds. Group two includes benzene hexachloride and the cyclodiene insecticides such as chlordane, heptachlor, aldrin, and dieldrin. Although flies may be tolerant of both groups simultaneously, they may also be developed individually with little or no cross resistance. Laboratory-produced strains generally require 15 to 20 generations of intensive selection to attain resistance to either group, but once established, the strain can be further selected with chemicals from the other group and in a few additional generations become resistant to these.

DDT and Related Compounds

Although houseflies, resistant to DDT, were shown in 1950 (5, 6) to be capable of detoxifying a large percentage of an applied dose of this compound through production of the dehydrochlorinated product, DDE, (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene), it was not until 1953 that the reaction was demonstrated to be enzymatically catalyzed (7). With the addition of glutathion to macerated adult flies, the breakdown was accomplished in vitro. A relationship between the capacity of the resistant flies to degrade DDT and the absence of such a mechanism in susceptible strains indicated that metabolism may indeed be an important factor in DDT resistance (8). The fact that this mechanism had a causal relationship to resistance rather than merely being a result of these flies living longer than the susceptible ones, was established by comparing the enzyme concentration in various strains obtained from different field and laboratory localities and pointing out the direct correlation between the degree of DDT tolerance expressed by the strain with the quantity of DDT-dehydrochlorinase within the flies. Additional evidence was later furnished (9) by observations showing that the effectiveness of the so-called DDT-synergists, which are structural relatives of DDT and partially revert the resistant flies to susceptibility, could be associated with in vitro experiments showing that these compounds serve as competitive inhibitors of DDT-dehydrochlorinase.

Throughout the larval life of the resistant housefly, DDT-dehydrochlorinase increases in titer until pupation. At this time, the enzyme concentration abruptly falls to about 50% of the maximum level attained. The resultant level then remains approximately static for the pupal period and through the adult stage (10). Distribution of the detoxication enzyme within the adult fly has been determined by pooling isolated tissues from many resistant individuals and then performing comparative assays on them (11). Distribution

appeared to be general, with the highest quantities located in the fat body, nervous tissue, and cuticle. The loss in detoxication potential at pupation and the occurrence of the dehydrochlorinating enzyme in the cuticle may be directly related. It appears that one of the major sites of DDT metabolism is present in those tissues which are early histolyzed during pupation. Proteolytic enzymes in the molting fluid may contribute to the decreased level of DDT-dehydrochlorinase through indiscriminate degeneration of protein constituents in the cuticular hypoderm.

Fractionation of the adult, resistant housefly yielded highly purified enzyme preparations (12). More recent studies (13) have culminated in the isolation of DDT-dehydrochlorinase from the resistant fly. The final preparations are reportedly monodisperse in the ultracentrifuge and essentially electrophoretically homogeneous.

Attempts to elucidate the origin of DDT resistance in the housefly in terms of this enzyme have been made (14). In order to learn if an adaptive role was operative, sublethal doses of DDT were topically applied to both susceptible and resistant imagines. Assays performed on breis prepared at varying intervals after treatment showed that no enhanced enzymic breakdown had been effected. Other experiments in which larvae were reared in a constant environment of low DDT levels or in higher concentrations of non-toxic analogs also failed to induce DDT-dehydrochlorinase.

In the course of these experiments, it was learned that a demonstrable level of DDT-dehydrochlorinase was present in the last instar of larvae of susceptible flies that had no prior history of DDT exposure. If a pattern of enzyme concentration during the life history of the susceptible fly parallels that shown in the resistant fly (10), it could follow that this detoxication mechanism has always been present; the process of selection, in this case, would merely have served to quantitatively increase it. The available methods of analysis are not sensitive enough to test such a hypothesis and, in fact, may be too crude to demonstrate a detoxication mechanism that has always been present in each life stage of the housefly. Evidence as to the natural function of DDT-dehydrochlorinase is completely lacking. Selection of flies with compounds other than chlorinated hydrocarbons (15) or by coinciding selection with timing of natural events such as pupation (10, 16, 17) have also produced increased DDT resistance and a concomitant elevation of enzyme level. These types of experiments preclude any mutagenic property of DDT and indicate that the toxicant serves primarily as a tool for selection.

To attain a full knowledge of insecticide resistance, natural tolerance as well as that acquired through selection or adaptation must be documented. The fate of DDT has been examined in the red-banded leaf roller, the Mexican bean beetle, certain grasshoppers (18), the milkweed bug (19), and various species of cockroaches (20, 21, 22, 23, 24). Similarly, reports on studies involving important vector species of resistant mosquitoes, flies, lice, fleas, and ticks have been numerous, and scattered records of metabolic studies associated with these insects have been recently assembled and annotated (2, 3, 4).

Hexachlorocyclohexane and the Cyclodiene Insecticides

Early attempts to show a metabolic involvement in resistance to toxaphene and chlordane succeeded in establishing indirect evidence that these compounds were degraded by the housefly. Topical applications made on resistant flies could not be quantitatively recovered or measured by bio-assay tests performed 4 and 24 hours after treatment (25). The nature of these changes has not been pursued.

In 1954, it was first reported (26) that the housefly could metabolize benzene hexachloride (BHC), but the ability to attack the gamma isomer, lindane, was characteristic of susceptible as well as resistant individuals. The rate of metabolism, however, proceeded at a faster pace in the more tolerant strains. Subsequent studies (27, 28) with the less toxic alpha and delta isomers of BHC pointed out that these compounds were also differentially destroyed by a series of eight different housefly strains. Further, the relative rates of breakdown could be correlated with the degree of resistance expressed by the strain. The rate of absorption varied in a like manner. These findings were confirmed (29, 30) and by utilizing paper chromatographic techniques and radioactive lindane, 11 different metabolites were demonstrated in extracts prepared from treated flies.

The first clue to characterization of BHC metabolism was the identification of pentachlorocyclohexene (31). This compound is not an end product, but is further metabolized in both susceptible and resistant flies. The metabolite does appear sooner and in larger quantities in the resistant fly. Recent studies on the fate of BHC have verified the presence of pentachlorocyclohexene in extracts of treated flies, and the identification has now been extended to include trichlorobenzene and tetrachloroadipic acid as breakdown products (32). These compounds all occur in small quantities and account for only a small percentage of the total absorbed dose. Although dehydrochlorination of lindane to pentachlorocyclohexene is reminiscent of the mechanism of DDT detoxication in the resistant fly, no metabolism of lindane occurs when it is presented as a substrate to purified preparations of DDT-dehydrochlorinase under optimal in vitro assay conditions.

The entire question of a metabolic role in BHC resistance has presented quite a paradox. A common factor in resistance to BHC and the Diels-Alder insecticides is strongly indicated by the cross resistance which has been repeatedly demonstrated in strains developed through exposure to any one of the lindane, heptachlor, chlordane, aldrin, dieldrin group. Although increased breakdown of BHC is clearly evident in flies tolerant to this class of compounds, the only metabolic changes that have been detected in the other compounds of the series result in intensifying their toxicity. Specifically, the only structural metabolic changes that have been found are epoxidation reactions. Conversion of heptachlor to its epoxide (33) and a similar alteration forming dieldrin from aldrin have been described. Thus, in the lindane, cyclodiene insecticide group metabolism of the insecticide is not unknown, but its real significance has yet to be ascertained.

NOTE: A portion of the section on carbamate resistance, metabolism, and synergism originally included in this paper has recently been published elsewhere (34) and has consequently been omitted from this review.

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CONTROL OF ANIMAL PARASITES WITH SYSTEMIC INSECTICIDES[1]

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To introduce our consideration of the systemic control of animal parasites, we quote from a paper (1) published two years ago, in which the authors, Radeleff and Woodward, discussed the past, present, and future of systemic parasiticides: "An intriguing concept of parasite control is that of attacking the offending organism in an indirect manner. Such an attack is based on the administration of a chemical, orally or parenterally, followed by absorption into the blood and transport to all parts of the body, there to be available to feeding parasites. For brevity, compounds used in this manner are called systemic parasiticides."

The concept of the systemic control of animal parasites is not new, for certain animal parasites have long been attacked in such a manner. A dramatic example of a systemic parasiticide is arecolin hydrobromide, which, when administered subcutaneously, results in the removal of taenoid tapeworms from the intestine. In contrast, however, the application of the concept in the control of parasitic arthropods is comparatively recent.

The arthropods that we have long sought to control include lice, mites, ticks, and biting flies, the latter including mosquitoes, horseflies, and stable flies. The most serious insect pests of livestock, however, are the screwworm, *Callitroga hominivorax* (Cqrl.), and the cattle grubs, *Hypoderma* spp. It is desirable to give brief outlines of the life cycles of these insects, since the damage they cause has given the greatest impetus to the search for systemic insecticides.

The screwworm is the common name of a species of fly that occurs from the southern United States into South America. The adult fly lays its eggs on the skin near a wound or infected body opening on cattle, sheep, and other domestic and wild animals. The larvae infest the wound and develop there, growing to two-thirds of an inch in length. Losses due to the deaths of infested animals run into millions of dollars annually in the southern United States. Hitherto, treatment has involved direct applications of insecticides to infested wounds.

Cattle grubs have a wider distribution and may be considered the most important livestock insects in North America. The adult flies lay their eggs, during the summer, on the hairs of the legs and lower parts of the bodies of cattle. The larvae penetrate the hide near the base of the hair and then migrate through the tissues until they reach the skin on the back of the animal. This migration takes several months, the larvae appearing in the back from February to May in the Guelph area. The larvae then penetrate the hide, thus

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providing breathing pores, and remain beneath the hide enclosed in cyst-like structures known as warbles. When mature, that is, when they are about an inch long, they squeeze out of the warbles, drop to the ground, and pupate. The adult flies emerge from the pupae in early spring, and the cycle is repeated. The migrating grubs doubtlessly affect weight gain and milk production, and encysted grubs damage the carcass and the hide. Until the advent of systemic insecticides, control measures were taken when the damage was complete, that is, by applying insecticides to the backs of cattle containing encysted grubs.

With this background, we can turn to a consideration of systemic insecticides. Serious consideration of their use against livestock pests dates back only about 30 years. The use of sulphur in this manner is an exception (2, 3, 4). In 1928, comprehensive experiments by Parman *et al.* (5) showed that external parasites of poultry, that is, lice, mites, and ticks, were not controlled by any systemic action of a large number of compounds then being sold for control of these pests. The compounds ranged from magnesium chloride to nux vomica.

Phenothiazine

Phenothiazine has often been referred to as the first promising systemic insecticide (6). Knipling (7), in 1937, administered it orally to cattle to determine whether it would destroy screwworms. It had no effect on the worms, but Knipling observed that manure from a treated calf was unfavorable for the development of manure-breeding maggots of various species. Bruce (8) shortly thereafter determined the minimum dose for horn-fly larvae in manure.

The compound was brought to notice again more recently, when Schwartz *et al.* (9) reported that free choice ingestion of phenothiazine by cattle had some toxic effect on migrating larvae of cattle grubs. The results of further tests, however, have been so variable that it is doubtful whether phenothiazine will ever be of great value (10, 11, 12). Its greatest value is as an antihelmintic (13, 14), although its use has been limited largely to the treatment of young animals because of its appearance in cows' milk and its presumed toxicity to man (15).

Studies on toxicity of phenothiazine to insects indicated that compounds that are in themselves comparatively nontoxic are activated or converted to toxic substances in the host or the parasite. In a study of the action of phenothiazine on the cockroach, Zukel (16, 17) showed that the compound was not toxic as a stomach poison, but was stored unchanged in the crop. It was lethal as a contact poison as a result of production of a leucothional conjugate in the haemolymph, the conjugate being formed by conversion of the phenothiazine in contact with or penetrating through the cuticle. Unconverted phenothiazine was not detected in the haemolymph. A similar conversion was noted for phenothiazone and thionol. Zukel concluded that the toxicity of phenothiazine might be due to the inhibition of cytochrome oxidase by the leucothionol conjugate. De Eds (18) and Thomas *et al.* (19) suggested that the permanent oxidation of the respiratory enzymes by the leucothionol–thionol

system occurred. Collier (20, 21) studied the effects of phenothiazine and related compounds on guinea-pig-liver catalase and beef-heart cytochrome oxidase, and reported that phenothiazine and phenothiazone were inactive, whereas leucophenothiazone, leucothionol, and thionol, the compounds containing hydroxy groups, were strong in vitro inhibitors. Collier and Allan (22, 23) later showed that the leucophenothiazone–phenothiazone system was a strong inhibitor of the oxidases and dehydrogenases of beef heart. They also commented on the action of phenothiazone on cholinesterase as a possible mode of action.

Botanical and Chlorinated Hydrocarbon Insecticides

The pyrethrins, although considered to be among the safest of insecticides, have received little attention as systemics and their use in this manner has been limited. Lindquist *et al.* (24) reported in 1944, that pyrethrins, when fed to rabbits, affected both the bedbug and stable flies that fed on the treated animals. They were also among the earliest to refer to the use of a chlorinated hydrocarbon as a systemic insecticide, stating that effective control of bedbugs was obtained with DDT. This was followed with reports by De Meillon (25) and Garnham (26) that gamma BHC (1,2,3,4,5,6-hexachlorocyclohexane) fed to rabbits controlled bedbugs, ticks, and mosquitoes.

The search for systemic insecticides was more highly organized in 1946, when the United States Department of Agriculture set up a screening program (27). None of the 200 compounds first tested were active. Then, in 1950, interest was concentrated on the chlorinated hydrocarbons by the report of Toledo and Saur (28) that BHC was systemically active against the human botfly, *Dermatobia hominis* (L., Jr.). Most of the chlorinated hydrocarbons were tested. Aldrin, dieldrin, lindane, and heptachlor were effective against deer flies and mosquitoes feeding on treated mice, but did not affect these insects on cattle (29). McGregor *et al.* (30) later found that aldrin, dieldrin, and lindane were effective against encysted cattle grubs but had little effect on migrating grubs. However, the use of chlorinated hydrocarbons as systemics was restricted by the high and persistent residues in the fat of treated animals (1, 31).

Organophosphorus Insecticides

The organophosphorus compounds were known, by this time, for their high level of insecticidal activity. Some had low mammalian toxicity. This combination, supported by the discovery that they were rapidly eliminated by mammals (32, 33, 34, 35, 36, 37), suggested their possible use as systemic insecticides.

O,O-dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate (Dipterex), *O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate (Diazinon), and *O,O*-dimethyl *O*-(3-chloro-4-nitrophenyl) phosphorothioate (Chlorthion) showed promise when tested with guinea pigs, but McGregor *et al.* (38)

reported that none appeared to affect migrating cattle grubs when fed to cattle. Drummond (39), in the latest report on the screening program, stated that *O,O*-diethyl *S*-(*p*-chlorophenylthio)methyl phosphorodithioate (Trithion) and 2,3-*p*-dioxanedithiol *S,S*-bis(*O,O*-diethyl phosphorodithioate) (Delnav) are ineffective as systemic insecticides.

The effectiveness of *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate (Trolene) against screwworms, stable flies, and migrating cattle grubs, when administered orally to cattle, was observed in 1955 at the U.S.D.A. laboratories at Kerrville, Texas, and Corvallis, Oregon (6, 40, 41, 42). A dosage of 100 mg/kg was sufficient for the control of cattle grubs. The compound has been assessed on more than 100,000 head of cattle in the United States and Canada, and is registered for this use in both these countries. At present, the material is applied by bolus, but applications in feed and by other methods are under investigation. Trolene has also been reported to be effective against mange mites (43, 44, 45), the sheep botfly (46), chicken lice (47), and helminths (48, 49).

Toxicological studies on Trolene in cattle, sheep, and other animals have been conducted by Radeleff *et al.* (50) and the Dow Chemical Company (51). Transient toxicity has been observed in some cattle treated at the therapeutic dose of 110 mg/kg, as in a field assessment conducted by one of us (A.A.K.). Diarrhoea, muscular fibrillation, increased respiration, bloating, and variously excessive salivation, sweating, and urination have been noted. Some observers have attempted to explain these symptoms as the result of an allergic reaction to, or an anaphylactic shock resulting from the death of grubs. The relationship between the symptoms and a probable anticholinesterase activity of the compound or its derivatives on the central and parasympathetic nervous systems, would be worth further investigation.

The metabolism of Trolene in cattle, rats, mice, and houseflies has been studied, with the P^{32}-labelled compound, by several workers, including Plapp and Casida (52). The suggested activation and detoxification of Trolene is shown in Fig. 1. They reported that, in rats, Trolene and its oxygen analog are hydrolyzed at two sites, and the primary hydrolysis products are rapidly excreted, 70% of the dose appearing in the urine as hydrolyzed metabolites. The first urine samples contained the highest percentage of phenyl phosphoric and phenyl phosphorothioic acids; in the later samples, dimethyl phosphoric acid preponderated. Dimethyl phosphoric acid, when fed to rats, was excreted unchanged. In cattle, the chloroform-extractable metabolite in the urine was predominantly the methyl hydrogen trichlorophenyl phosphorothioate. Characterization of the hydrolysis products of Trolene in the housefly showed that the primary site of hydrolysis was at the phosphorus–oxygen–phenyl bond.

Two of the three initial pathways of breakdown, that is, the phosphorothioate oxidation (I) and the hydrolysis of the phosphorus–oxygen–phenyl bond (II), were already known, for example, with Diazinon as reported by Robbins *et al.* (53). The hydrolytic attack at the alkoxy groups (III), yielding a stable monalkyl aryl phosphorodiester, was a new suggestion. Plapp and Casida also found that both Trolene and its oxygen analog were susceptible to hydrolysis

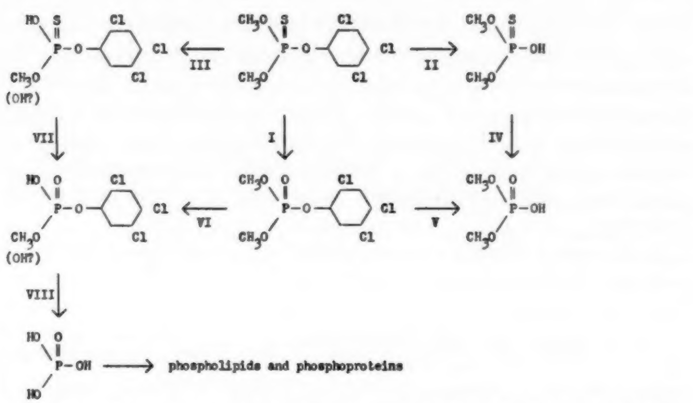

FIG. 1. Metabolic pathways of Trolene in the rat, cow, and housefly, as suggested by Plapp and Casida.

at the phosphorus–oxygen–methyl bond (III and VI), as well as to cleavage of the phosphorus–oxygen–phenyl bond (II and V), the degree of attack varying with the chemical and biological systems and conditions involved. A similar study by Casida *et al.* (54) on Phosdrin, a dimethyl carbomethoxypropenyl phosphate, showed that this compound was rapidly metabolized by the cow, and excreted as dimethyl phosphoric acid.

The principal toxic metabolite of Trolene appears to be the oxygen analog. Plapp and Casida were not able to recover it as an in vitro metabolite in either cattle or houseflies, or from the in vitro incubation with rumen fluid. Indirect evidence of its formation was obtained from the inhibition of blood cholinesterases, as well as of fly-brain cholinesterases in Trolene-treated animals, despite the low activity of Trolene as compared with that of the oxygen analog. Similarly, March *et al.* (55, 56) and O'Brien (57, 58, 59) suggested that the principal toxic analog of malathion in animals is the oxygen analog, malaoxon. Plapp and Casida found that the oxygen analog of Trolene is about 2000 times as potent an inhibitor of fly-brain cholinesterase in vitro as Trolene.

Plapp and Casida suggested that the selectivity of Trolene might be explained by the observation that hydrolysis of the compound by houseflies was predominantly at the phenyl group (II and V), in contrast with rats, in which it was at the methyl group (III and VI). This recalls again the investigations on the metabolism of malathion by March *et al.* and O'Brien, which strongly suggested that the compound is not toxic to mice and cattle because of the vigorous hydrolytic degradation of either malathion or malaoxon at the carboxylic ester link. This process outstrips the malaoxon accumulation. In the cockroach, however, activation is more rapid than hydrolysis.

An interesting aspect of the metabolism of the organophosphorus compounds is the activity of the rumen fluid of cattle. Cook (60) has shown recently that the rumen fluid attacks some of the thiono compounds, but not any of the thiol compounds. Parathion, for example, is apparently not toxic when fed to

cattle, in sharp contrast to its effect on other species, such as sheep, goats, and dogs. Cook found that the in vitro action of rumen fluid on parathion, measured by a cholinesterase method, was rapid. The activity of the fluid on Trolene was examined by Plapp and Casida. There was no conversion to the oxygen analog, and primary hydrolysis was at the phosphorus–oxygen–methyl group to yield phenyl phosphoric acid. Some dimethyl phosphorothioic and dimethyl phosphoric acids were formed, suggesting oxidative metabolism as well as hydrolysis.

McGregor *et al.* (38) classed *O,O*-diethyl *O*-(3-chloro-4-methyl-7-coumarinyl) phosphorothioate, formerly known as Bayer 21/199 and now as Co-ral, with Dipterex, Diazinon, and Chlorthion as compounds that are not systemically active when fed to cattle. Brundrett *et al.* (61) and Graham (62) reported later that the compound was highly active when applied dermally. It is the second organophosphorus compound to be registered in Canada and the United States for the systemic control of cattle grubs. An 0.5% suspension is sprayed on cattle at 1 gallon per animal. When applied at the recommended dosage, it causes only a mild, transitory reduction in blood cholinesterase activity.

We are unable to find any reports of studies on the activity of rumen fluid on Co-ral. Lindquist *et al.* (63) reported that, in rats, the compound was rapidly metabolized and excreted when administered orally. Krueger *et al.* (64) found four metabolites in the urine, after oral, subdermal, and dermal treatment of rats. After oral treatment, diethyl phosphorothioic, diethyl phosphoric, and phosphoric acids were present, together with the de-ethylated derivatives of the compound or its oxygen analog, or both. After subcutaneous administration, phosphoric acid was not recovered. Dermal applications did not lead to the detection of the de-ethylated derivatives. After a study of the metabolism of the compound in a cow, Krueger *et al.* concluded that the main difference in its metabolism, as compared with that of other dialkyl aryl phosphates and phosphorothioates, was that complete degradation to phosphoric acid appeared to occur more rapidly.

Finally, concerning the organophosphorus compounds, the following have shown promise in the systemic control of migrating cattle grubs: *O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate (Dimethoate); *O*-(4-*tert*-butyl-2-chlorophenyl)O-methyl methyl phosphoramidothioate (Dowco 109); *O,O*-dimethyl *S*-2-(ethylthio)ethyl phosphorodithioate (Bayer 23/129); and, *O,O*-dimethyl *S*-2-(ethylsulphinyl)ethyl phosphorodithioate (Bayer 23/453). They are being assessed in the field, and presumably the toxicity and metabolism of each are being studied. One of them, Dimethoate, was included by Hewitt *et al.* (65) in a series of 19 carbamoyl alkyl phosphorodithioates for a study of structural–activity relationships. Dimethoate was the most active. The *O,O*-dimethyl compounds were superior to the *O,O*-diethyl analogs, but the higher *O,O*-alkyls were uninteresting. In the *O,O*-dimethyl series, the unsubstituted carbamoyl group was less than 1/20th as active as the methyl carbamoyl.

Comments on Future Work

Many aspects of the systemic control of animal parasites await investigation or further study. These include the metabolic pathways in treated animals and parasites, including activation and detoxification, toxicology of the compounds in the treated animals, the complex host–parasite relationships, the means by which the toxicants reach the parasite, mode of action on the parasite, structural–activity relationships, and many others. The procurement of knowledge on these subjects requires the co-operative effort of the entomologist, parasitologist, chemist, physiologist, toxicologist, animal husbandryman, and other specialists.

Addendum in Answer to Questions from the Floor

Dr. E. H. Colhoun (Science Service Laboratory, London, Ontario) asked about the acceptance of systemic insecticides. Mr. Peterson replied that most veterinary entomologists have welcomed and readily accepted the systemic insecticides. On the other hand, many toxicologists and veterinarians are most cautious in expressing their approval.

In reply to a second question, Dr. Kingscote expressed his opinion that the future for systemic insecticides is very promising. There is no doubt that certain organophosphorus compounds, for example, are efficient in destroying a number of insect parasites within the bodies of their hosts or through the medium of the medicated blood upon which a number of ectoparasites feed. To destroy parasites in this manner, before they do extensive damage to the host's tissues or act as vectors of disease, would be a great step forward in preventive medicine. Much more yet, however, may have to be learned about the action of systemic insecticides upon insects, animals, and man before they can be used with full confidence. Another factor, too, regarding the general acceptance of the new insecticides will be the cost of their production. The systemic insecticides have shown such great promise, as Mr. Peterson has pointed out, that there seems little doubt that, with so many investigators studying them, any problems that exist now will be resolved in due course and such insecticides will have a very definite place in our economy.

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COMPARATIVE TOXICOLOGY OF SOME ORGANOPHOSPHORUS COMPOUNDS IN INSECTS AND MAMMALS[1]

R. D. O'Brien

A. The Need

From the beginning of time, man has been interested in killing his enemies without endangering his friends, and this aim is the origin of selective toxicology. This paper is concerned with only one aspect of this problem: that of killing one species of animal and sparing another.

As far as agriculture is concerned, there are two further arbitrary subdivisions that may be set up. *Firstly*, we want to kill insects, but we want to do it with compounds safe to man and livestock. We want, in more general terms, to kill arthropods and spare chordates, i.e. to distinguish between phyla. In view of the apparent enormous differences between these phyla, the problem should be easy, but of course it is not. Our target should be to make it possible eventually to outlaw the use of all insecticides that are hazardous for man or livestock.

Secondly we often want to distinguish between different arthropods, so that we kill only the undesirable ones. Experience has shown that indiscriminate destruction of insect populations often results in a rapid resurgence later on, and may lead to a greater preponderance of the undesirable species than existed in the first place. Alternatively, new pests, formerly of too low an incidence to be important, may reach outbreak proportions. Our target should be to develop a series of safe insecticides of varying spectra of activity, so that the economic entomologist may choose the extent and type of his kill.

In this paper, we shall first see what selective organophosphates are available. Next we shall examine the fundamentals of selectivity. We shall then pick out one or two of the fundamental mechanisms and illustrate how knowledge about them has led to the development of new compounds of predictable selective toxicity. Finally we may generalize briefly upon the sorts of attack upon the problem that ought to prove most profitable.

Because this paper tries to present a fundamental rather than a primarily economic approach, no apologies are made for paying attention to compounds which exhibit interesting but uneconomic selectivity, e.g. compounds fatal to man and harmless to insects.

B. The Known Selective Organophosphates

The early postwar organophosphates such as TEPP and DFP were relatively non-selective. In the last few years an increasing number of selective compounds have been developed, all prepared without knowledge of basic principles, but by extensive synthesis and screening. There is the possibility that this

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shotgun technique is the most effective one; but it is to be hoped that the next 20 years will prove that it is not.

Table I shows the insect:mammal toxicity ratio for some selective organophosphates. Where possible, the toxicity given is for intraperitoneal administration to the rat and topical application to the fly. The compounds listed show a very striking selective toxicity; whereas one group is almost exclusively insecticidal, the other is almost exclusively "mammalicidal".

TABLE I

Toxicity ratio (insect/mammal) of organophosphates

Insecticidal (all P = S type)		Mammalicidal (all P = O type)	
Co-ral	150	Tetram (=Amiton)	0.005
Malathion	68	Schradan (=OMPA)	0.01
Diazinon	88	Ro 30412	0.0037
Dimethoate (=12,880)	1400	Nu 1250 (a carbamate)	0.00059
Viozene (=Trolene =Dow ET14)	1000		

NOTE: The figures (which are only approximate) represent the ratio of the LD_{50} for the rat (intraperitoneal) to the LD_{50} for the housefly (topical), both on a mg/kg basis.

The first question to be answered is: what are the causes for these remarkable differences? A full enough answer to this question would perhaps enable us to design new compounds, whose selective toxicity will be predictable.

C. Principles of Selective Toxicity

Let us consider the various steps that can occur when a poison is applied to an animal (of any phylum). All possible steps will be included although some will of course be lacking in certain cases.

Figure 1 is a diagram of an animal being poisoned. It will be noted that there are six quite different stages shown; a seventh step that may occur is storage.

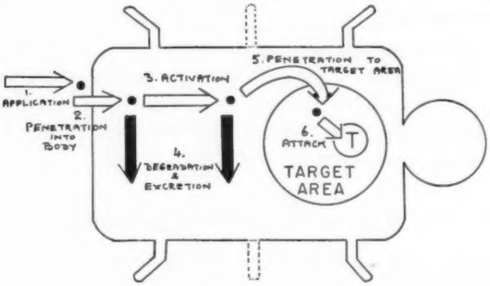

FIG. 1.

Stage 2 can be avoided by injecting the poison instead of applying topically. Stage 3 is required for the phosphorothionates and phosphoramidates only. We shall work on the assumption that the target for all organophosphates is the same, and we shall assume even more tentatively that it is in fact cholinesterase. However, the argument will not be seriously affected if it is not.

If we have made the diagram complete enough, then we can say this: that if an organophosphate kills species A and not B, then these species differ in one or more of these stages. All we have to find out is which one is involved.

Now we shall dispose briefly of stages 1 and 2, because they are rather obvious, and say only a little about stage 6, because we have so little data. We shall then consider 3, 4, and 5 in detail.

Application

This factor is of trivial fundamental interest, but in practice is used to obtain a kind of "selective toxicity": appropriate spraying and protective equipment ensure that the insecticide is applied to the insect and not to the gentlemen driving the tractor. A more interesting use is with plant systemic insecticides: by poisoning the inside rather than the outside of the plant, we ensure that only phytophagous insects are killed.

Penetration into Body

For some insecticides, it has been shown that resistance is connected with slow cuticular penetration (1). Such effects have not been shown for organophosphates. On the whole, the insect is rather better protected than the mammal. One would expect some quite radical differences between, say, the housefly and the American roach, with respect to penetration of the integument. Krueger has in fact found such a difference with malathion, but the difference appears to have little effect upon toxicity (2).

Target

This is not the place to debate whether cholinesterase is the target of organophosphate poisoning in insects. In the following discussion it will be assumed that it is, but there is still some uncertainty on this point.

In most respects insect cholinesterase appears to behave like mammalian true cholinesterase. However, Van Asperen (3) has shown for DDVP, and Mengle (2) has confirmed for DFP and paraoxon, that although mammalian cholinesterase in vitro slowly recovers from inhibition by these compounds, housefly cholinesterase does not. In spite of this, housefly cholinesterase in vivo recovers rapidly in the survivors from organophosphate poisoning. This paradox is still under study. It will of course be of tremendous importance if we find that cholinesterase is fundamentally different in insects and mammals. We could perhaps use this difference to design compounds that would inhibit only insect cholinesterase.

The remaining steps will be dealt with in some detail. They are of two types: those concerned with metabolism and those concerned with penetration. First we have to consider the alterations of the poison inside the animal body; secondly the problem of penetration of the active material into the target area.

Metabolic Differences

Several years ago we started the study of malathion, because it was an important insecticide of low mammalian toxicity. We chose the cockroach, the housefly, and the mouse for our type animals. Malathion has an LD_{50} for the cockroach or fly of about 10 mg/kg, for the mouse of about 1000 mg/kg.

Malathion, being a phosphorothionate, is a poor anticholinesterase, but is "activated" by tissues of the mammal and the insect. This activation involves conversion to malaoxon, which is a potent anticholinesterase. The most important finding resulted from a very simple experiment (4). Malathion or malaoxon was added to suspensions of insect and to mammalian tissue preparations, then samples were removed at intervals and added to cholinesterase in order to assay the malaoxon.

With liver slices, added malaoxon was degraded extremely rapidly. It is therefore not surprising that added malathion was not effectively activated, since the malaoxon produced would be broken down. With cockroach gut preparation added malaoxon was degraded only very slowly. As one might expect, when malathion was added, activation resulted in a steady accumulation of malaoxon, and therefore a steady increase in anticholinesterase material.

It seems likely therefore that when malathion is injected into the roach, the insect converts it readily to malaoxon, which it cannot degrade easily. As a result, the insect accumulates enough malaoxon to kill itself. The mouse, however, degrades malaoxon so readily that a toxic dose is never accumulated. Krueger (2) has confirmed that this is the case in the intact animal: after poisoning with malathion (30 mg/kg) the malaoxon level in the mouse reaches 0.1 mg/kg, then rapidly declines; in the roach it reaches 0.4 mg/kg and is persistant.

Krueger has recently investigated malathion metabolism in insects using unusually hot P^{32}-labelled compounds, and separating the metabolites by column chromatography (2). It is a curious fact that the insecticide is poisonous to the American roach (LD_{50} about 10 mg/kg) but not to the German roach (LD_{50} about 120 mg/kg). It was hoped that this striking difference would be attributable to some metabolic difference.

The degradation products from malathion metabolism in these two insects and in the fly were first examined in detail and three surprising things were found:

(1) The insects produced nine water-soluble degradation products, rather than the one or two expected from the literature (Fig. 2).

(2) At the LD_{50}, all of the insects had degraded about one-half of the applied dose in 30 minutes—yet roaches die several days after treatment. This suggests that the insecticide attacks its target promptly, but the effects of that attack are slow in appearing.

(3) All three insects degraded malathion to the same extent and to the same products, whose relative proportions varied, however.

Since degradation was not more extensive in the non-susceptible German roach, perhaps activation could be less extensive, i.e. the German roach might survive because it manufactured little malaoxon from malathion. But this hypothesis was disproved by showing that malaoxon levels after treatment with malathion were the same in both insects.

We are therefore forced into the conclusion that the difference between the German and American roach must lie in penetration to the target, or in attack

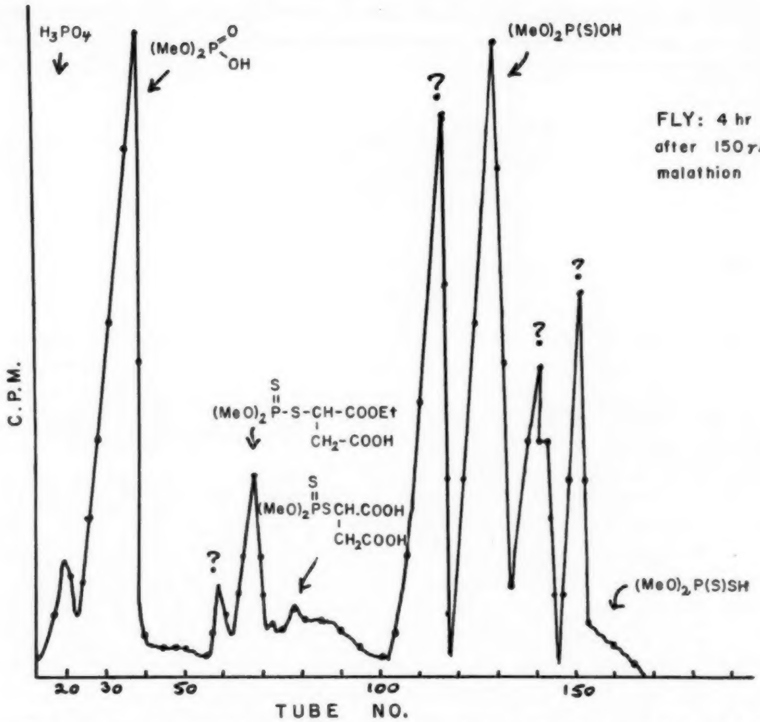

FIG. 2. Chromatographic analysis on an ion-exchange column of metabolites in the bodies of flies 4 hours after topical application of radioactive malathion, 150 mg/kg.

on the target, or in the nature of the target. But we have as yet no evidence to offer on this point.

Oppenoorth, in Holland, recently studied various strains of flies with varying resistance to parathion (5). On analysis (using an anticholinesterase assay) he found that the activation product (paraoxon) accumulated only in the susceptible strain. There are three possible explanations why the resistant insects fail to accumulate paraoxon: (1) they do not activate parathion rapidly; (2) they degrade parathion rapidly; or (3) they degrade paraoxon rapidly. Oppenoorth next observed that the resistant flies were also resistant to topically applied paraoxon. Therefore the resistance must be due to rapid paraoxon degradation.

Recently O'Brien and Wolfe (2) examined Co-ral metabolism in tissues of various animals. In brief, they found that Co-ral is degraded quite rapidly in tissues of the cow and the rat, which are non-susceptible species, and very slowly in those of the cattle grub, the housefly, and the mouse, which are susceptible species.

Now Krueger (6) has shown that Co-ral is degraded in the cow and the rat primarily at the P–O-coumarinyl link but also a little at the ethoxy link (see

below) (Fig. 3). Presumably one of these is the weak link which is attacked readily by the cow and rat, and poorly by the mouse and fly. Figure 3 also

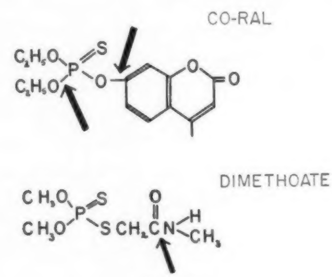

FIG. 3.

shows the selective insecticide Dimethoate and the weak link as far as the mammal is concerned. We may guess that it is the presence of this group that confers selective toxicity, although we have as yet no proof.

In all the cases for which we have a reasonable explanation of selective toxicity it seems that selectivity is associated with what we may call a "weak link" in the molecule, which only the resistant insect can readily break. In the case of malathion, the weak link is probably the carboxyester group.

These weak links are the primary object of initial hydrolytic attack. Hydrolysis of an organophosphate almost always yields an anionic phosphorus-containing fragment which is a poor anticholinesterase. We should expect, in fact, that all anionic compounds will be poor anticholinesterases because:

(*a*) Organophosphates operate by attacking cholinesterase. This attack is electrophilic, and therefore the phosphorus atom must be rather positive to get a good effect.

(*b*) Anionic groups, being negatively charged, are strong electron repellers, and therefore make the neighboring phosphorus atom negative, therefore the molecule is a poor cholinesterase inhibitor.

We can now suggest a principle for making selective insecticides to kill organism A and not B. First find out what links can be broken by B but not by A. Next introduce this link into a potent or potentially potent anticholinesterase, in such a way that the breakage of this link introduces an anion into the phosphorus-containing moiety. B will then detoxify the molecule readily while A will not, so the compound will be selectively toxic to A.

The weak link in malathion is the carboxyester group, which we thought must imply that this link is degraded readily in the mammal and not in the insect. We therefore incorporated the link into some new compounds (7) and thereby produced new insecticides which we predicted would have low mammalian toxicity; these compounds are shown in Table II.

The prediction was correct. It will be noticed that the phosphorothionates show a better toxicity ratio than their phosphate analogues. This is to be expected, due to what we might call an "opportunity factor". The presence of the thiono group means that time has to be spent in oxidizing it to produce

TABLE II
New insecticides designed for selectivity

		Toxicity ratio (fly/mouse)
Acethion	$(EtO)_2P(S)SCH_2COOEt$	136
Acetoxon	......P(O)...........	63
Prothion	$(EtO)_2P(S)SCH_2CH_2COOEt$	25
Propoxon	......P(O)...............	8
Methyl methprothion	$(EtO)_2P(S)SCH(CH_3)\text{-}CH_2COOMe$	33
	(Cf. malathion	68)

a potent compound, and this time gives an opportunity for any detoxifying processes to take place. Therefore differences due to different detoxification rates are shown best with phosphorothionates. Studies with acethion, the most promising of these compounds, showed an interesting difference from malathion. Malathion is toxic to the housefly and to the roach; acethion is toxic to the housefly but not to the roach. Further examination showed that acethion was degraded relatively rapidly by the non-susceptible mouse and roach, and not at all by the susceptible fly (7). Once again there appears to be an excellent correlation between degrading capacity and resistance to poisoning.

Let us now sum up our ideas on cases where metabolic differences are the cause of selective toxicity. Firstly, this selectivity always seems to be in favor of the mammal. Secondly, in all cases studied, we have been able to explain the fact that mammals are insensitive to certain anticholinesterases by showing that they degrade them rapidly. Thirdly, a similar explanation also explains the selective toxicity in the insect world in some cases, but not in the case of malathion.

The theory of the weak link may help towards our first goal—the invention of new non-hazardous insecticides; but may go only part way to fulfilling the second goal—the development of compounds that will select between insect species.

Penetration Differences

We next will consider, very briefly, the other kind of selective poisons: those which kill mammals but not insects. Attention was drawn to this problem by the long-standing acetylcholine paradox. It had been known for some time that acetylcholine was toxic to mammals but not to insects. This had led some investigators to believe that the acetylcholine – cholinesterase – choline acetylase system is not important in insects. However, an observation was made by Hoyle that insect nerves were protected from potassium ions by a sheath. We wondered if the barrier that protected the nerves would also protect them from other ions, such as acetylcholine. We postulated an ion barrier, present in the insect but not in the mammal, which protected the nervous system (and perhaps other systems) from toxic ions.

There are by now several bits of evidence that favor this hypothesis (8, 9, 10, 11). In fact three classes of compounds may be enumerated where insecti-

cidal activity is adversely affected by the barrier. These classes are (I) all ionized compounds; (II) many ionizable compounds; (III) at least one very polar but un-ionized compound.

(I) Ionized Compounds

There is now a variety of evidence, electrophysiological (11), enzymological (8), and histochemical (10) that acetylcholine is ineffective as an insecticide purely because of its failure to penetrate to the nervous system. In general it is true that all ionized anticholinesterases are extremely poor insecticides, even if they are excellent mammalicides. The mammal:insect toxicity ratio is usually between 500 and 2000 for organophosphates containing a quaternary nitrogen.

For some completely inexplicable reason, the sulphonium ion derivative of Isosystox does not obey this rule closely. Its mammal:insect toxicity ratio is only about 5 for the housefly, and the highest ratio noted was only 100, for the American roach (9).

(II) Ionizable Compounds

The most important of these are the compounds containing a tertiary nitrogen, and the best known is Tetram (Fig. 4). This figure points out two

		(Mouse/roach) toxicity ratio
Tetram:	$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$	220
Quaternarized Tetram:	$(C_2H_5O)_2P(O)SCH_2CH_2\overset{+}{N}(C_2H_5)_3$	2350

FIG. 4.

things: The unfavorable toxicity ratio of Tetram, and the way in which this ratio is enormously increased by quaternarizing the nitrogen. We have some fairly extensive data on the penetration of the cockroach nerve cord by Tetram, as a function of pH (2). The evidence is that the unionized molecules of Tetram penetrate, but the ionized ones do not.

Now the fraction of the molecule that is ionized at a given pH is given by the pK_a of the compound. The pK_a is easy to measure, and one can make intelligent guesses at how the pK of a compound will be modified by introducing new substituent groups near it. Bases with high pK_a's are primarily ionized at physiological pH, and therefore will be poor insecticides, since only the un-ionized fraction is effective in the insect. There is therefore an upper limit upon the pK which the insecticide inventor can allow himself. With a pK of 7, only half of the insecticide is effective within the insect. With a pK of 8, one tenth is effective. With a pK of 9, one hundredth is effective, and so on. Tetram has a pK_a' of about 8.4 from which one may calculate that only 4% or 1/25 is un-ionized at pH 7.

(III) Un-ionized Compounds

Under this section it is merely desired to record the fact that we now believe that schradan is ineffective against most insects because of a penetration problem. The potent anticholinesterase metabolite, now thought to be not schradan N-oxide but hydroxymethyl schradan (12) is a polar but not ionized

compound. It fails to penetrate to the roach nerve cord (13). We have yet to prove that it does penetrate to the nerve cord of such susceptible insects as the aphid and squash bug. If we can show this, we may be on the way to exploring the variations in nerve permeability within species. We may even learn to use this variation to kill unfriendly and save friendly insect species.

D. Future Approach

The final question is: in the light of what we already know, what will be the most effective way to develop the battery of new selective insecticides which we need?

The most wasteful way will be to continue the present method of extensive relatively undirected synthesis followed by screening tests.

As far as utilization of the "weak link" theory is concerned, we should first pick the animals we want to kill and those we want to spare, and examine them to find suitable link-breaking enzymes (probably all hydrolases) which are relatively active in the type to be spared, and of low activity in the type to be killed. We can then build in appropriate links to otherwise potentially potent anticholinesterases, and get selective poisons.

As far as the ionization problem goes, we must first study the properties of the insect ion-barrier in many important species of insects. We should certainly be able to tell the chemist within what permissible p*K* limits he should work in designing ionizable compounds. If there are differences between insect species in their ion barriers, we may be able to make compounds to kill certain species only, by modifying the p*K* in (for instance) organophosphates containing a tertiary nitrogen. In this way, we would make compounds sufficiently ionizable that they were ineffective against insects with good ion barriers.

These suggestions are based upon the utilization of only a few pathway differences. Further intensive fundamental studies will undoubtedly show us how to utilize many other differences as well. Above all, let us advance reasonably, instead of randomly.

Acknowledgments

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ENZYME RELATIONSHIPS IN THE ACTION OF ORGANOPHOSPHORUS INSECTICIDES[1]

B. N. SMALLMAN

Most of what we say in this symposium is based, directly or by implication, on the hypothesis that the toxicity of an organophosphorus insecticide depends on its ability to inhibit the enzyme acetylcholinesterase. In our discussions on the differential toxicities of these compounds, and on the development of resistance to them, we are concerned with mechanisms that potentiate or reduce their effectiveness as inhibitors of acetylcholinesterase; we are concerned also with the physiological consequences of inhibition of this enzyme, and the extension of the hypothesis to their ovicidal action. The hypothesis supposes further that after inhibition of acetylcholinesterase, its substrate, acetylcholine, accumulates in the nervous tissue and produces the ultimate toxic effect. The enzymic relations underlying our subject therefore are those that govern the hydrolysis and synthesis of acetylcholine. The metabolism and physiological significance of acetylcholine in insects is fundamental to our understanding of the action of the organophosphorus insecticides, and it seems appropriate to begin this symposium with a brief review of the current status of the anticholinesterase hypothesis from this point of view.

Earlier doubts about the presence in insects of the elements for the hypothesis have now been dispelled. During the past few years, unequivocal evidence has been found for the presence of acetylcholinesterase, acetylcholine, and the enzymic system for its synthesis in the nervous tissue of insects (1). All three elements have been demonstrated also in the eggs of insects, and they usually, though not always, appear coincidentally during embryonic development (2, 3). Colhoun (4) has determined the distribution of acetylcholine in the roach and demonstrated its presence in the peripheral as well as the central nervous system; the highest concentrations were found in the head and thoracic ganglia. Finally, the properties of the acetylcholinesterase (5) and the mechanism of acetylcholine synthesis (6) appear to be essentially the same in insects as in mammals.

However, the presence of acetylcholine and the mechanisms for its metabolism in insects do not establish its functional significance. The well-established insensitivity of insects to injected acetylcholine argues against its functional significance and against the anticholinesterase hypothesis. A clue to this anomaly was afforded by Twarog and Roeder (7), who demonstrated that removal of the connective tissue sheath of the sixth abdominal ganglion of the roach reduces the concentration at which synaptic transmission is affected by many pharmacological agents, including acetylcholine. Furthermore, O'Brien (8) showed that acetylcholine and other highly ionized compounds were not

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hydrolyzed by the semi-intact roach preparation and deduced from this evidence the existence of an "ion barrier". An indication of the functional significance of the acetylcholine system was obtained by Van der Kloot (9), who observed the remarkable coincidence between, on one hand, the disappearance and renewal of electrical activity in the brain of *Cecropia* in diapause and, on the other hand, the decline and rise in cholinesterase activity, the level of an acetylcholine-like substance, and of choline acetylase. In a study of the relation between nervous activity and acetylcholine in the thoracic nerve cord of the roach, Colhoun (10) showed that intense nervous activity induced by eserine, with or without stimulation, was accompanied by a significant increase in the acetylcholine content of the thoracic ganglia. However, when this method was applied to the well-defined synaptic pathway in the sixth abdominal ganglion, significant accumulations of acetylcholine were not detected in the ganglion despite evidence that the presynaptic fibers were active and the postsynaptic response was blocked. In general, the evidence supports a relation between acetylcholine and nervous activity in insects but fails to indicate clearly its role as a synaptic mediator.

Curiously, little attention has been given, in insects or in mammals, to the corollary of the anticholinesterase hypothesis; that is, that inhibition of cholinesterase results in an accumulation of acetylcholine in nervous tissue. Fisher and I (11) have recently reported that, after lethal doses of anticholinesterases, acetylcholine does in fact accumulate above normal levels in the housefly, the roach, and the mouse, and this accumulation occurred (in the housefly) with all of several anticholinesterases tested. Moreover, we showed that the degree and duration of the supranormal levels of acetylcholine varied with the degree and duration of cholinesterase inhibition and with toxicity. With TEPP for instance, a sublethal dosage applied to houseflies gave a transitory increase in acetylcholine levels, a dose that caused about 80% mortality gave somewhat higher levels of acetylcholine which remained above normal for several hours, and a dose that gave 100% mortality resulted in acetylcholine values nearly double the normal value and these high values persisted for still longer intervals before returning to the normal level. We proposed that the areas under such curves for the rise and fall of acetylcholine levels might provide better correlations with the toxicities of organophosphate insecticides than the degree of cholinesterase inhibition at some arbitrary time after treatment.

However, it appears that the factors involved in these changes in acetylcholine levels are too complex to lend themselves to such a simple correlation. There are indications that changes in the rate of acetylcholine synthesis, and generalized morbid changes affecting acetylcholine levels, may also occur. In the roach, for instance, Colhoun (12) has shown that treatment with TEPP induces a sharp and transitory increase of acetylcholine, associated with violent nervous activity; this is followed by a slow increase to very high levels and an ultimate decline to zero. The latter phase was associated with morbid changes in the roach tissues. Treatment with DDT, which of course does not inhibit cholinesterase, failed to show the sharp initial increase in acetylcholine but

showed the large later increase and ultimate decrease in almost the same degree and duration as treatment with TEPP. This important finding suggests that, if acetylcholine is indeed the lethal agent, it exerts its effect during the initial excitatory phase and that the later large increases are secondary, resulting from morbid changes. They may, in fact, result from an accelerated synthesis of acetylcholine due to the breakdown of permeability barriers and the increased availability of substrates to enzymes for the synthesis of acetyl coenzyme A and acetylcholine. In other words, morbid changes in the tissues may permit the synthesis of acetylcholine to proceed more nearly at the rate observed under optimal conditions in vitro, and the activity of the synthesizing enzyme, choline acetylase, has been shown to be high in insects (6). The final decrease of acetylcholine to very low levels or its complete disappearance may then result from further tissue breakdown which brings acetylcholine in contact with cholinesterase. However, when cholinesterase remains completely inhibited, acetylcholine continues to increase for periods as long as 36 hours after treatment of houseflies with parathion (11).

Clearly, if we are to test our hypothesis and understand the relations between cholinesterase inhibition, accumulation of acetylcholine, and death, we must establish what levels of cholinesterase inhibition result in supranormal levels of acetylcholine. The recent findings of Van Asperen (13) and Colhoun (12) throw doubt on the finding that acetylcholine increases above normal only when cholinesterase is completely inhibited. They have shown that secondary inhibition of cholinesterase by free organophosphate during homogenization may cause greater inhibition of cholinesterase than that which actually occurs in vivo, and that the true in vivo inhibition is revealed only when the enzyme is protected by excess substrate. It seems likely, therefore, that our findings with TEPP (11) indicate that acetylcholine can accumulate to above normal levels after sublethal doses that induce only a partial inhibition of cholinesterase. We need to know also the degrees and durations of the supranormal levels of acetylcholine that are associated with death.

There are many indications that the various anticholinesterase insecticides do not act uniformly, despite the common factor of cholinesterase inhibition. For instance, the effect of DFP on acetylcholine levels appears anomalous among cholinesterase inhibitors in that after a short initial increase in acetylcholine the values do not increase but remain constant (11). This suggests that DFP, in addition to inhibiting cholinesterase, also interferes with acetylcholine synthesis. Attempts to demonstrate an inhibition of choline acetylase activity by in vitro methods have so far failed, but Winteringham *et al.* (14) have obtained evidence for an in vivo derangement of the synthesis mechanism.

Evidence obtained from the ovicidal effects of organophosphates appear to support the hypothesis. Insect eggs treated with these compounds at an early stage of embryonic development continue to develop normally until just before hatching and then die (15, 16). This delayed action appeared inconsistent with the hypothesis until it was shown (15) that housefly eggs contain little or no detectable acetylcholine during early development but that there is a sudden and spectacular rise in acetylcholine content just prior to hatching.

Cholinesterase activity also appears at about the same time in untreated eggs but is completely inhibited in eggs exposed to parathion soon after oviposition. It follows that if acetylcholine is the ultimate toxic agent in organophosphate poisoning, then no mortality would be expected during early development when this substance is absent. But late in development inhibition of the normal controlling enzyme, cholinesterase, exposes the embryo to the high acetylcholine concentrations then present and death ensues.

From time to time doubts have been expressed that acetylcholinesterase is indeed the vital target for the anticholinesterase insecticides. Recently, Van Asperen (13) has pointed out that ali-esterases are inhibited to the same degree as acetylcholinesterase in insects treated with O,O-dimethyl-O-2,2-dichlorovinyl phosphate (DDVP) and has warned against committing ourselves to the anticholinesterase hypothesis until the functions of these ali-esterases are known. Winteringham (17) has also pointed to biochemical derangement other than cholinesterase inhibition. He has also shown that the hydroxylamine derivative, PAM, which protects mammals against DFP, relieved the initial paralysis in houseflies treated with this compound, but the flies nevertheless proceeded to die.

These results clearly indicate that we must remain alert for explanations other than the inhibition of cholinesterase for the toxic effects of organophosphorus insecticides. But at this stage the bulk of the evidence accumulated for insects, backed by the impressive evidence from mammals, still favors the anticholinesterase hypothesis as the best basis for interpreting the toxic action of organophosphorus insecticides.

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PHYSIOLOGICAL EVENTS IN ORGANOPHOSPHORUS POISONING[1]

E. H. Colhoun

Organophosphorus insecticides may be divided into two broad groups, depending upon the initial toxicity of the applied compound. The first group represents the direct acting compounds of which tetraethyl pyrophosphate (TEPP) is a well-known example. The second class of compounds differs from the first in that conversion of the parent compound to a more toxic poison takes place within the organism. This is seen in the oxidation of O,O diethyl O-*p*-nitrophenyl phosphorothioate (parathion) to diethyl *p*-nitrophenyl phosphate (para-oxon). In vertebrates both TEPP and para-oxon are powerful inhibitors of cholinesterase (ChE). Coincident with the inhibition of ChE, accumulations of acetylcholine are found in conductive tissue together with significant malfunction of conductive processes. These three events are intimately related and they appear to have fundamental importance in the physiology of the nervous and muscular systems. After treatment with organophosphorus compounds death or survival of the organism will, therefore, depend upon the degree of disruption of these systems and the resultant effects of their malfunction upon other vital processes. In vertebrates death is rapid for compounds such as TEPP and para-oxon inhibit the true ChE of the central, autonomic, and somatic nervous systems. Paralysis ensues, followed by coma and suffocation. The vertebrate physiologist is not concerned with events beyond this. Certain peculiarities are found in insects treated with organophosphorus insecticides which warrant an understanding of basic physiology and a realization that insects have a physiology of their own. Therefore, let us examine some of the effects of organophosphorus poisoning in insects. Mostly I would like to deal with my own work, some of which is recent and not yet published.

The Occurrence of Acetylcholine, Cholinesterase, and Choline Acetylase in Conductive Tissue of the Roach

First, I want to establish the occurrence of ACh, ChE, and choline acetylase (ChA) in the conductive tissue of the American cockroach, *Periplaneta americana* L. At this point I would like to emphasize that many hypotheses have been propounded as to the function of ACh in insects, without adequate knowledge whether this ester and related enzymes did indeed occur within the tissue under experimentation. The results given in Table I show that ACh, ChE, and ChA are found in the central and peripheral nervous system. ACh and ChA have a similar tissue distribution with the highest concentration in brain and ganglionic nervous tissue. The amounts of ChA are better correlated with the amounts of ACh than those of ChE. Iyatomi and Kanehisa (1) found

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TABLE I

The distribution of acetylcholine, cholinesterase, and choline acetylase in conductive tissue of the roach

Tissue	ACh (μg/g)*	Q_{ChA} (mg/g/hr)†	Q_{ChE} (mg/g/hr)
Brain	143.6±4.0‡	50.6	137.7±15.4
Brain + suboesophageal ganglion	135.2±2.0	53.0	153.4±21.2
Ventral cord	63.2±4.5	10.6	270.4±16.3
Thoracic cord	79.0±6.1	11.4	221.3±15.9
Thoracic ganglia	95.4±4.3	20.8	331.8±12.4
Thoracic connectives	31.3±2.4	2.6	238.7±18.5
Abdominal cord	65.2±1.6	6.2	187.5±10.1
6th abdominal ganglia	63.0±1.5	18.0	314.9±20.6
5th leg nerve	1.21 μg per 60 nerves	2.0	176.5±26.8
Cercal nerves	1.43 μg per 60 nerves	3.6	150.8±30.3
Coxal muscle	0	0	0
Flight muscle	0	0.08	?
Heart	0	0	0
Blood serum	0	0	?
Blood cells	0	0	?

*Colhoun, E. H. J. Insect Physiology, **2**, 108 (1958).
†Colhoun, E. H. Nature, **182**, 1378 (1958).
‡Standard deviation.

somewhat similar distributions of ChE in the roach although they stated that the brain contained the highest amount of enzyme. The amount of ChE in the nervous tissue of the roach is much higher than that reported for grasshoppers (2). So far no attempt has been made to establish the occurrence of aliphatic and aromatic esterases in similar regions of conductive tissue of the roach. These enzymes have been reported to occur in the nerve cord (3). I have been unable to demonstrate the occurrence of ACh, ChE, and ChA in denervated roach muscle. Some hydrolysis of ACh was found with muscle breis when using Warburg microflasks, but these results were different from the typical hydrolysis curves obtained with homogenates of nervous tissue. The evidence given in Table I shows that the nervous system of the roach has the components of the ACh system, which can be affected by organophosphorus insecticides. I would like to stress that acetylcholine is the only choline ester found so far in the central nervous system of the roach (4) and that there is no evidence of any other neurohormone to equal the concentration or distribution of ACh. Dr. Chefurka and I have attempted to demonstrate the distribution in the roach of other possible neurohormones, such as adrenaline, noradrenaline, and 5-hydroxytryptamine, as yet we have been unsuccessful and unable to support the evidence of Ostlund (5), who found adrenaline and noradrenaline in other insects.

The Function of Acetylcholine in the Nervous System of the Roach

Next, I would like to mention the function of ACh in the nervous system of the roach for in this lies an important aspect of organophosphorus poisoning. Hopf (6) showed that injected ACh could not be considered toxic to insects. This and other observations led to the conclusion that ACh may have no functional significance in insects. However, Hoyle (7) and Roeder and associates (8, 9) obtained evidence that the nervous system of insects was protected by a

sheath which prevented the easy penetration of the cations, K^+ and ACh. O'Brien (10) termed the protection an "ionic barrier". One questions whether the barrier preventing the penetration of ACh into the central nervous system of the roach is entirely different from the brain blood barrier of vertebrates.

We decided to approach the problem from 'within'. Colhoun (11, 12) showed that there was a correlation between nervous activity and increases of ACh in the isolated eserinized nerve cord of the roach. ACh did not diffuse readily out of the nerve cord. This evidence is somewhat analogous to the vertebrate central nervous system, for here ACh is found in the cerebrospinal fluid rather than in the blood. It can be concluded from these experiments that there is an association between nervous activity, ACh, and ChE, for eserine is a known anticholinesterase. There is no precise evidence that ACh is a synaptic mediator in the nervous system of the roach. This problem must await more precise experimentation with intracellular electrodes on the association of function with specific ChE inhibition and in this respect we have some encouraging results.

The Effect of Insecticides upon Acetylcholine Levels and the Inhibition of Cholinesterase

In work parallel to that mentioned above, Smallman and Fisher (13, 14) and Lewis and Fowler (15) obtained evidence that treatment of insects with organophosphorus compounds resulted in high accumulations of ACh, and Smallman and Fisher (13, 14) obtained correlations between the inhibition of ChE and increases in ACh. I decided to investigate the correlations of symptoms, nerve function, ChE inhibition, and ACh in the nerve cords of roaches poisoned with TEPP (16, 17). After topical treatment of male roaches with a lethal dose of TEPP, 5 μg per roach, two distinct and separate increases of ACh were found in the thoracic nerve cord. The first occurred at early poisoning during hyperexcitability; the second, much greater, increase of ACh occurred after the roaches were prostrate and when the nerve cord was electrically silent. This latter observation was at first disturbing for it indicated that the increase of ACh might have no relation to nerve function. However, Tobias *et al.* (18) and Van der Kloot (19) found increases of ACh in insects without toxic inhibition of ChE. We treated roaches with a lethal dose of DDT (17) and found that there was a high increase of ACh at late poisoning when the roaches were prostrate. ChE was uninhibited. At late poisoning this increase in ACh was similar to that found in TEPP-treated roaches. At this time in TEPP-treated roaches, the inhibited ChE was slowly recovering activity. In a recent experiment we injected roaches first with pyridine 2-aldoxime methiodide (PAM) and then topically treated them with 5 μg TEPP. The initial increase of ACh was prevented (Fig. 1), but the second high increase of ACh occurred when the roaches were prostrate. These roaches died but the typical TEPP symptoms were prevented before the roaches became prostrate. More will be said about this later. I suggest that these increases of ACh at late poisoning are more suggestive of synthesis rather than a release and accumulation of ACh resulting from nerve function and the inhibition of ChE.

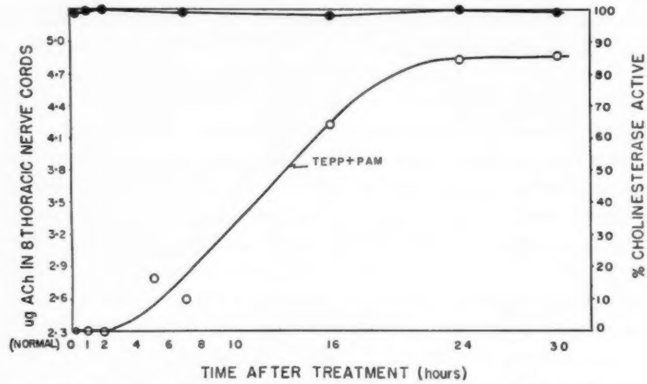

FIG. 1. ACh levels and ChE activity in thoracic nerve cords of roaches pre-treated with PAM, followed 5 minutes later with a lethal dose of TEPP.
○ = ACh level. ● = ChE activity.

In substantiation of this hypothesis we compared the ACh content of ganglia and connectives of thoracic nerve cord of roaches at 25 hours after treatment with TEPP and DDT (17). In each treatment the results showed a parallel increase in the ACh content of ganglia and connectives. The ratio of ACh in ganglia to that in connectives was 1:3, which was identical with the ratio in normal thoracic nerve cord. After TEPP- and DDT-treatment the intracellular distribution of ACh in thoracic nerve cord was found to be the same when determined by the technique of differential centrifugation. We also attempted to determine whether the high increase of ACh found at late poisoning was due to accelerated synthesis, particularly as Lewis (20) found that breis, made from houseflies treated with DDT, showed a higher rate of endogenous ACh synthesis than breis of untreated flies. We found no evidence of increased endogenous ACh synthesis in breis of nerve cords of roaches treated with TEPP and DDT. This work was extended with more precise methods, using suitable cofactors for the synthesis of ACh (21). Again there was no evidence that breis prepared from nerve cords of treated roaches showed a higher rate of synthesis than breis of normal nerve cords. Addition of TEPP and DDT to the medium did not promote a faster rate of ACh synthesis. This evidence, taken as a whole, indicates that the increase of ACh, found in nerve cords of roaches at late TEPP- and DDT-poisoning, is not the result of abnormal synthesis.

I would now like to discuss briefly the increases of ACh in the thoracic nerve cords of roaches at early TEPP treatment (17). We found that ChE was completely inhibited at the time when the roaches were showing only very slight tremors. Subsequent experiments showed that this result was in part due to the further inhibition of ChE by TEPP during homogenization of the nerve cords. Free TEPP was found to be present in the nerve cords. Homogenization of the nerve cords in saline containing ACh prevented the in vitro inhibition by TEPP. This technique, termed substrate protection of ChE, was used by Van Asperen (22) to determine the probable in vivo inhibition of ChE

in flies poisoned with organophosphorus insecticides. In our work the use of this technique gave better correlation between symptoms, ChE inhibition, and the increase in ACh. Further work is being carried out on this aspect with more emphasis on nerve conduction and transmission.

We extended the technique of substrate protection with ACh to nerve cords of roaches topically treated with para-oxon, parathion, and bis(dimethylamino) phosphorus anhydride (Schradan). The latter two compounds are converted to a more toxic poison within the insect. The results are summarized in Table II.

TABLE II

Effect of addition of ACh at homogenization on ChE inhibition of roach thoracic nerve cord after topical treatment with parathion and para-oxon

Time after treatment, hours	b_{30}*, normal thoracic cord	b_{30}, thoracic cords from treated roaches	b_{30}, thoracic cords from treated roaches + excess ACh	% protection by ACh in homogenate	b_{30} of normal cords + treated cords
(*a*) Parathion					
0.5 (no symptoms)	77.1 ± 7.5†	73.1 ± 3.8	72.3 ± 6.5	0	129.0 ± 9.6
1 (slight symptoms)	73.0 ± 8.6	69.4 ± 5.8	54.8 ± 9.2	0	130.5 ± 11.3
2 (hyperactive-prostrate)	68.5 ± 11.4	43.8 ± 8.3	38.4 ± 5.4	0	85.6 ± 8.2
3 (prostrate)	70.3 ± 5.1	0	0	0	67.1 ± 10.3
(*b*) Para-oxon					
0.25 (just prostrate)	67.5 ± 6.1	0	0	0	39.1 ± 4.0

*b_{30} refers to the μl of CO_2 evolved in 30 minutes after correction has been made for non-enzymatic hydrolysis.
†Standard deviation.

We found that substrate protection of ChE by ACh was unnecessary when homogenizing thoracic nerve cords after treatment of roaches with parathion. No inhibition of ChE was found with schradan-treated roaches. Substrate protection was required for ChE determinations after treatment with para-oxon for, as found for TEPP, free inhibitor was present in the nerve cords. Substrate protection was not required for ChE determinations after treatment with parathion, a finding different from that obtained by Van Asperen (22). Because Van Asperen used whole insects in his experiments we investigated the possibility that an inhibitor might be present in roach blood. The results given in Table III show that after topical treatment with parathion the blood of roaches contained a detectable amount of inhibitor. The amount of inhibitor increased with time after treatment. It is of interest that, although schradan did not produce toxic symptoms in the roaches, a small amount of an anticholinesterase was found in blood at 5 to 6 hours after treatment. We conclude from these results that homogenization of whole roaches after treatment with parathion or schradan would have resulted in an in vitro inhibition of ChE. Since 'free inhibitor' was not detected in the thoracic nerve cord after treatment of roaches with parathion I suggest that parathion is an excellent compound with which to determine the probable in vivo inhibition of ChE in the absence of substrate protection.

TABLE III

The presence of inhibitors in the blood of roaches after treatment of roaches with parathion and schradan

Time after treatment, hours	b_{30}* of normal thoracic cord	b_{30} of normal thoracic cord + 0.5 ml blood	b_{30} of normal thoracic cord + 0.5 ml blood + ACh in homogenate
(*a*) Parathion			
1 (no symptoms)	76.5±5.0†	62.1± 9.6	70.3±6.4
3 (prostrate)	72.0±7.3	24.8± 4.2	72.5±8.5
(*b*) Schradan			
1 (no symptoms)	72.5±8.2	71.0± 3.9	69.3±7.4
5–6 (no symptoms)	74.5±4.1	54.6±10.9	78.4±6.1

*b_{30} refers to the μl of CO_2 during 30 minutes after correction has been made for non-enzymatic hydrolysis.
†Standard deviation.

Blood Substances after Treatment of Roaches with Insecticides

The study of blood can give useful indications of events occurring within tissues. We examined the blood of TEPP- and DDT-treated roaches at early poisoning. The results given in Table IV summarize some of the work by myself and Dr. Chefurka. The most distinct difference between the modes of action of TEPP and DDT was the finding that ACh appeared in the blood of TEPP-treated roaches (16). The appearance of the other substances in the blood of the treated roaches might be indicative of a common stress factor resulting in the release of hormones from glands, for two of these hormones are known to occur in the corpus cardiacum and corpus allatum. These substances have not been identified chemically, although the corpus cardiacum hormone is thought to be similar to noradrenaline (23). Perhaps these substances produced some of the toxic blood symptoms described by Sternburg and Kearns (24) and Sternburg *et al.* (25).

TABLE IV

The occurrence of hormones in the blood of roaches topically treated with TEPP and DDT

Substance*	Blood of prostrate TEPP-treated roaches	Blood of DDT-treated roaches†
Acetylcholine‡	+	−
Corpus cardiacum hormone‡	+	+
Corpus allatum hormone	+	+
A (5-hydroxytryptamine ?)	+	+
B Unknown	+	+
C Unknown	+	+

†Roaches first held at 35° C for 20 hours; then transferred to 15° C and bled at 2 hours when prostrate.
*Identification by partition chromatography and by differential bio-assay‡.

Acetylcholine and Cholinesterase in Roaches Treated with TEPP and PAM

In conclusion I would like to refer back to our work with PAM, which has been used successfully by vertebrate physiologists in conjunction with atropine as an antidote in organophosphorus poisoning. PAM is thought to reactivate

inhibited ChE by dephosphorylating inhibited ChE, thus forming a phosphorylated oxime. Our interest was stimulated by the observation of Winteringham (26), who found that flies pretreated with PAM and then treated with a lethal dose of diisopropyl phosphorofluoridate (DFP) did not show typical organophosphorus symptoms although they died. We injected roaches with PAM and 5 minutes later gave a lethal dose of TEPP. The roaches became prostrate but did not show the usual striking TEPP symptoms. We examined the ACh content of thoracic nerve cords and determined the activity of ChE. The results are shown in Fig. 1. It is noteworthy that the initial increase of ACh found with TEPP-treatment alone did not occur. The second high rise of ACh was found. The ChE of the thoracic nerve cord was found to be uninhibited (Fig. 1 and Table V*a*) but these results are not conclusive for there is

TABLE V

The ChE activity of roach thoracic nerve cord after treatment of roaches with PAM and TEPP

Time after treatment	b_{30}*, normal thoracic cords	b_{30} of thoracic cords from TEPP-treated roaches	b_{30} of thoracic cords from roaches treated with PAM and TEPP	b_{30} of thoracic cords from normal roaches treated with PAM combined with cords from TEPP-treated roaches
(*a*) Treated with PAM for 5 minutes followed by a lethal dose of TEPP				
44 min	82.0±9.6†	0	73.5±4.2	—
24 hours	75.6±4.9	8.2±2.3	86.0±7.1	—
(*b*) Treated with the tertiary analogue of PAM 5 minutes followed by a lethal dose of TEPP				
45 min	82.0±9.6	0	0	—
(*c*) The in vitro reactivation of TEPP-inhibited ChE by PAM in normal roach cord				
45 min	145.8±10.1 (10 cords)	0	—	109.6±15.4 (10 cords)

*b_{30} refers to the liters of CO_2 evolved during 30 minutes after correction has been made for non-enzymatic hydrolysis.
†Standard deviation.

the possibility that in vitro reactivation of ChE occurred during the manometric determinations of ChE activity. Evidence of this was found (Table V*c*) when homogenates of nerve cords of roaches treated with PAM were combined with homogenates of nerve cords of TEPP-treated roaches. Seventy-five per cent of the activity of the TEPP-inhibited ChE reappeared during the course of the Warburg experiments. At least one can conclude that in vitro PAM reactivated the inhibited ChE of nervous tissue of the roach. Therefore, if PAM can penetrate into the nerve cord in vivo, as our results suggest, reactivation of inhibited ChE should occur within the insect. The tertiary analogue of PAM, prepared in the London laboratory by Dr. E. Y. Spencer, was ineffective in preventing the inhibition of ChE of roaches treated with TEPP, as shown in Table V*b*.

Death was not prevented in roaches treated with TEPP and PAM, and indeed one might feel that this observation negates the hypothesis (14) of the mode of action of organophosphorus poisoning in insects. I feel that any such conclusion is premature for some of our preliminary work with PAM at the

LD_{50} to LD_{100} level of TEPP has shown definite indications of protection against TEPP poisoning. One questions whether PAM or other reactivators of inhibited ChE would protect against a high level of organophosphorus in vertebrates, particularly as atropine is used in conjunction with the reactivator. Roaches are insensitive to atropine, but on the other hand there is little known about the mechanism of insect neuromuscular transmission. Atropine may block the effect of ACh at the vertebrate neuromuscular junction. Winteringham (26) has obtained evidence that DFP not only inhibits ChE in flies, but that in vivo it may interfere with ACh synthesis. This observation was obtained after DFP-treatment by following the in vivo rate of C_{14} acetylation of choline. The assumption can be made, therefore, that death in flies, pretreated with PAM and then given a lethal dose of DFP, may be due to a lack of ACh rather than to the inhibition of ChE. These excellent experiments serve to point out the need to obtain more basic facts about insect physiology and biochemistry. A knowledge of fundamental mechanisms is an a priori requirement upon which to raise the edifice of toxicology.

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THE OVICIDAL ACTION OF ORGANOPHOSPHATE INSECTICIDES[1]

E. H. Smith and A. C. Wagenknecht

In considering the insecticidal action of organophosphates, analogy is made to the more extensive findings on mammalian toxicity. Here it is generally agreed that acetylcholine (ACh) serves as the conductor of nerve impulses at some synaptic and at neuromuscular junctions and that the enzyme cholinesterase (ChE) prevents its accumulation by rapid hydrolysis. Presumably then, when ChE is inhibited, ACh accumulates thus subjecting the organism to excessive levels of ACh which induce toxicity. The specific disturbance which finally accounts for death is not known.

In considering this mode of action in insects a number of objections arose. First, it had not been established that the elements required by the hypothesis were common to insects. Secondly, there was the striking insensitivity of insects to injected ACh. Thirdly, there was the evidence that other esterases which might play vital roles were also inhibited. The first two objections have been largely resolved and the pertinent evidence has been admirably reviewed by Smallman (1). Finally, the possibility should not be overlooked that the inhibition of still other enzymes might account for the toxic action of organophosphates.

It is equally natural in considering ovicidal action to draw on evidence derived from hatched forms. In drawing such an analogy, basic differences in the biological systems of eggs and hatched forms must be recognized. Chief among these is the absence of a differentiated nervous system in early embryonic stages. In addition, embryonic development likely involves processes for utilization of stored energy and differentiation of the organism which are not common to hatched forms. On the other hand, the nearly mature embryo possesses some of the same vital systems found in newly hatched forms such as nervous, respiratory, and circulatory systems. Thus ovicidal action might involve either a vital process common to hatched forms, or one unique to the process of embryonic development.

The present paper reviews the evidence relating to ovicidal action of organophosphates and considers the modes of action likely involved. The matter of species susceptibility and some practical considerations in the use of organophosphate ovicides has been reviewed in an earlier paper (2).

Characteristics of Organophosphate Poisoning

Early studies on ovicidal action of organophosphates reported that death occurred in advanced stages of embryonic development and this observation was variously interpreted. It was concluded that parathion was not a true ovicide, the larvae being killed when they were chewing out of the chorion by

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contact with surface residue or toxicant dissolved in the chorion (3, 4, 5). It is now established that the action is ovicidal, eggs being susceptible at all stages of development (6, 7).

Whether or not continued development of eggs treated in early stages is characteristic of organophosphate poisoning is of cardinal interest in studies of mode of action. Death in early stages has been reported by Lord and Potter (8) and Schwartz (4) using TEPP and parathion, respectively. In both cases dosages greatly in excess of those employed in practice were used. Other investigators (7, 9, 5, 10) have reported continued development as a characteristic of organophosphate poisoning. Whether development continues after treatment may depend on the concentration of toxicant employed, but unfortunately the variations in experimental methods preclude valid comparisons on this central point.

Susceptibility of Eggs at Various Stages of Development

If ovicidal action involves specific physiological systems such as the nervous system it would seem reasonable that susceptibility would be restricted to the stage of embryonic development when this system is present. This was not the case, eggs being susceptible at all stages of development as cited above. While no striking break in susceptibility was evident it seemed likely that some degree of differential susceptibility would occur during the course of embryonic development and that such evidence might provide clues to the mode of action involved.

In attempting to establish differential susceptibility, distinction must be made between physiological susceptibility and the dosage–mortality relationship as influenced by such factors as permeability of the chorion. As both the embryonic material and the chorion are undergoing simultaneous change their separate influences on mortality are difficult to assess. Furthermore, it is not known how the toxicant gains entry to the egg. Lethal quantities may be acquired from toxicant applied to the chorion or by exposure to vapor. If the toxicant gains entry as vapor in the respiratory exchange of gases, then the respiratory rate would also be a variable requiring consideration. A fivefold change in rate of oxygen consumption in the course of embryonic development of the peach tree borer has been reported (7). Permeability of the chorion undergoes changes of perhaps equal magnitude as suggested by Salkeld and Potter (11).

In the case of the peach tree borer susceptibility to parathion varied with the type treatment employed (7). When the toxicant was applied directly to the chorion susceptibility increased with age, the reverse being true in exposure to vapor. Small increases in susceptibility with increasing age were found in several species treated with hexaethyl tetraphosphate (HETP) (11). The matter of susceptibility has been considered further by Potter *et al.* (12). The essential feature of these various studies is that in no case were striking differences shown which might suggest when the vulnerable system appeared, as the differences could conceivably be accounted for without change in physiological susceptibility.

Respiratory Studies

In attempting to establish mode of action, effort is made to relate toxic symptoms to effect on a biological system. Toxic symptoms such as hyperactivity, convulsions, and paralysis which are readily observed in hatched forms do not apply in the case of eggs. It seemed likely that interference with a vital system would be reflected in the gross rate of oxygen consumption of the organism. Studies along this line have been reported by two investigators and their findings are in general agreement (10, 7). Treatment in early or late stages of development causes no marked change in oxygen consumption until near the normal time of hatching. At this time the rate begins to decline and death ensues several days after the normal time of hatching. Although this evidence failed to pinpoint the time when lethal action took place it verified the gross observation that development continued normally following treatment. With respect to systems involved, this picture, as it applies to early treatment, presents two possibilities:

(1) A system is attacked at the time of treatment but this system plays no vital role until late stages of development.
(2) The system attacked is not present in early stages, retention of the toxicant being required until the appearance of the vulnerable system.

While continued development following treatment has been cited for a number of other ovicides the situation is not analogous to that reported here. The dinitro cresols (11) and petroleum oils (13), widely employed as ovicides, cause immediate change in the rate of oxygen consumption, the degree of change being closely correlated with dosage. On the contrary, parathion treatment induces no immediate change and within broad limits the respiratory pattern is not altered by changes in dosage. It is also of interest to note that by contrast to eggs, hatched forms show a sharp rise in respiration following organophosphate treatment (14).

Occurrence of ACh and ChE

The disclosure that organophosphates inhibit ChE of the insect nervous system led to studies on the occurrence of the enzyme and its substrate, ACh. Unfortunately the occurrence and role of these interacting components have largely been considered independently. This likely stems from the fact that the omnipresence of ACh in insects remained in doubt for some time, its positive characterization being a rather recent development (15, 16). Furthermore, the techniques involved in assay of the two are different. Aside from these factors, information pertaining to eggs was further limited because of the doubtful applicability of findings based on hatched forms and difficulties in obtaining adequate quantities of experimental material.

The evidence on occurrence of ChE in insect eggs is more extensive than for ACh. In nine species representing four orders the pattern of ChE occurrence is reported to be broadly similar (17, 18, 8, 19, 20, 21). The enzyme first appears shortly after the mid-point of incubation and increases progressively until hatching. In relation to embryonic development the evidence is insufficient to warrant generalization. Potter *et al.* (6) established seven stages of

embryonic development in *Pieris brassicae* L., one for each day of development. ChE appeared in the fifth stage, the first in which the nervous system appeared fully developed and presumably functional. Similar findings were reported by Staudenmayer (20) and Chino (17) using eggs of the silkworm. Earlier occurrence, 20–30 hours before blastokinesis, is reported in the rice stem borer and cabbage army worm by Chino and Yushima (18). These workers concluded that in these species there was no correlation between appearance of ChE and development of the nervous system.

Chino (17) has investigated the occurrence of both ACh and ChE in eggs of the silkworm. The synthesis of ACh is reported shortly after blastokinesis, which occurred at 5 days (10-day incubation period). In vitro synthesis coincided with actual appearance of ACh in vivo. This in turn coincided with the appearance of ChE so that presumably ACh decomposition was possible from the time of its initial appearance. This evidence altered earlier reports from the same source that ACh was not detected in eggs of the silkworm (18).

The simultaneous appearance of ACh and ChE as reported in the silkworm is not found in the rice stem borer and cabbage worm according to Yushima (22). Here ACh activity was first detected at the neuroblast stage increasing rapidly up to blastokinesis followed by a gradual increase until hatching. In both species the occurrence of ACh preceded ChE by 40 or more hours. This lag in appearance of ChE is cited by the authors in explaining the accumulation of ACh during stages of embryonic development. In the housefly ChE appeared at 7 hours thus preceding ACh, which was detected at 9 hours (12-hour incubation period) (9). No generalized pattern regarding the sequence of these events is apparent from the evidence cited. It seems likely that as the course of embryonic development in insects is rather generalized the same might apply to associated biochemical components. By such rationale the protective mechanism, ChE, would be expected to precede or coincide with the appearance of potentially harmful ACh.

Qualitative Studies of Esterases

Qualitative differences in esterases of mammals have been recognized and systems proposed for their characterization (23). Differentiation of esterases has been based largely on substrate specificity, response to excess substrate, and selective inhibition. Efforts to fit insect esterases to the classifications proposed for mammalian esterases have met with limited success. The importance of more precise differentiations of insect esterases became evident following the disclosure that ChE's of various insect species differ in their susceptibility to various organophosphate compounds (24). While later studies have shown that insects present a complex of closely related esterases much more information is needed in this area so vital to mode of action studies (25, 26).

Before evidence derived from hatched forms can be applied to ovicide studies it must be known whether the complex of esterases remains the same throughout the various life history stages. Casida (26) cited changes between stages of the housefly based on substrate specificity and selective inhibition. A limited

comparison can be made between the esterases of milkweed bug heads (25) and eggs of the same species (19). The similarity in substrate specificity pattern with two substrates suggests that the same esterases are involved.

The differences in esterases in relation to ovicidal susceptibility has been considered in two species, the peach tree borer, highly susceptible to ovicidal action of parathion, and the milkweed bug, which is unaffected at practical concentrations. ChE from both sources reacted with ACh to give bell-shaped activity/substrate concentration curves while benzoylcholine was hydrolyzed at very low rates in both cases. By contrast, activity–pS curves using acetyl-β-methylcholine showed differences in the esterases from the two species. The significance of this difference to ovicidal susceptibility will be discussed later. In summary it can be said that more comprehensive understanding of the biochemical nature of esterases is needed before valid comparisons can be made between species of eggs or between eggs and hatched forms.

The ineffectiveness of ovicidal treatment to eggs of the milkweed bug might be due to failure of the toxicant to penetrate to the site of action, or insensitivity of the esterases to inhibition. As qualitative differences had been shown the latter seemed a likely possibility and in vitro inhibition studies were conducted to provide additional evidence. The values required for 50% inhibition (IN 50) were approximately the same for both species, being of the order of 3×10^{-3} M. These values are high as might be expected if metabolic conversion of parathion did not occur in vitro. However, the similarity of these values in the two species suggests that lack of ovicidal effectiveness against the milkweed bug is likely due to failure of the toxicant to reach the site of action. This could be accounted for by a barrier to its entry or to rapid breakdown following its entry. Conceivably the latter process might be ineffective in protecting against quantities of toxicant introduced in vitro. It seems most likely that the lack of ovicidal effectiveness of parathion to the milkweed bug is due to failure to penetrate and accumulate at a site of action, this view being supported in part by high susceptibility of newly hatched nymphs to parathion.

Effect of Treatment on ChE and ACh

Extensive studies on the effect of ovicidal treatment on ChE have been conducted by Smith and Wagenknecht (19). These involved eggs of the peach tree borer and milkweed bug representing extremes in ovicidal susceptibility. Eggs were treated by exposure to filter paper impregnated with parathion (0.035%). Treatment of this type resulted in 85% and 15% inhibition of ChE respectively in the peach tree borer and milkweed bug. Treatments made in early stages of development before the appearance of ChE resulted in a high degree of inhibition in the susceptible species in late stages of development when the enzyme is normally present. The significant features of these findings are: (1) the relationship established between ChE inhibition and ovicidal susceptibility, (2) the delayed action effect whereby early treatment ultimately results in ChE inhibition. The latter point suggests that the inhibitor is retained in the egg until the appearance of the enzyme. Similar treatments using eggs of the housefly resulted in complete inhibition of ChE (9). Inhibition by eserine has

also been reported in eggs of the silkworm and the IN 50 value is comparable with values for fly head ChE (20).

While ChE inhibition had been demonstrated in ovicidal action its effect on ACh levels was unknown. Evidence on this point was essential in considering the involvement of ChE in the mode of action, as toxicity is presumably directly attributable to ACh accumulation rather than ChE inhibition. This point was investigated by Mehrotra and Smallman (9) using eggs of the housefly. Both ChE and ACh levels were determined following treatment with parathion. With complete inhibition of ChE a 50% increase in ACh occurred by the 11th hour thus supporting the view that toxicity may be due to ACh accumulation. A phenomenal increase in ACh occurred from its initial appearance until the 11th hour and it is likely that these levels would have been still higher at 12 hours when hatching normally occurs.

Additional evidence regarding the accumulation of ACh following inhibition of ChE is provided by Chino (17). In these studies eserine was employed as an inhibitor in eggs of the silkworm. At 8.5 days (10-day incubation period) the ACh level in the presence of eserine was over 100% above normal.

The evidence cited would appear to explain the delayed action whereby treatment at any time finally induces death in late stages of development. Accordingly the inhibition of ChE removes the mechanism controlling ACh levels. For a time after the initial appearance of ACh, the levels in the absence of ChE remain below the toxic threshold. With continued activity coincident with hatching the levels rise still higher until the toxic threshold is reached.

In treated eggs of the peach tree borer (7) and silkworm (10) death occurs several days after the normal time of hatching and embryonic activity can be observed during this interval. The levels of ACh occurring throughout the course of embryonic activity in treated eggs would be of interest. Do ACh levels reach a peak at the normal time of hatching or does continued nervous activity result in still higher levels?

In similar studies on hatched forms it has been noted that high ACh levels following treatment may decline before death. This observation led Wintringham and Harrison (27) to suspect that high ACh levels are not the cause of death. Later studies by Smallman and Fisher (28) cited levels of ACh as high as 260% above normal following complete inhibition of ChE. The decline at the time of death was attributed to release of normally bound ACh which was then hydrolyzed by reactivated ChE. This evidence would seem to resolve the anomaly posed by low ACh levels at the time of death. Based solely on the relationship between ChE inhibition and ACh levels the evidence from both eggs and hatched forms does not invalidate the ChE inhibition concept as it relates to toxic action.

Other Esterases

The possibility exists that in ovicidal action other esterases and indeed other enzymes are inhibited which play a vital but as yet unknown role; in such a case, the observed inhibition of ChE would be merely coincidental. This possibility was proposed by Lord and Potter (8) based on: (1) the occurrence of phenyl-

esterases in all stages of embryonic development; (2) inhibition of these esterases by in vitro treatment with TEPP; (3) death of treated eggs in early stages of embryonic development before the occurrence of ChE. This evidence provides a valid challenge to the ChE inhibition concept and is deserving of careful assessment.

The first point to be examined is the suitability of TEPP for ovicidal studies. In selecting it these workers reasoned that due to its relative instability to hydrolysis, toxic action would likely occur shortly after treatment and before the occurrence of ChE in eggs treated in early stages of development. This assumption is open to question and will be considered after first commenting on the effectiveness of TEPP.

Practically speaking TEPP is not an ovicide, being ineffective at the concentration generally employed in practice.[2] Its LD 50 value against eggs of the peach tree borer is approximately 50 times the concentration employed in practice. By contrast parathion, a recognized ovicide, is employed at 24 times the LD 50 value. Considering the unique instability of TEPP and its relative ineffectiveness as an ovicide it can hardly be considered typical of organophosphate ovicides.

Despite this objection some pertinent questions are raised by the data pertaining to the relationship between concentration of toxicant and stage of development at which death occurred (Table I). The embryonic stages referred to are fully described in the reference cited. At high concentrations it was possible to obtain complete kill in early stages of development. As the dosage was reduced the proportion of eggs continuing development increased. In assessing these results it is assumed that massive dosages likely involve modes of action which do not occur under conditions of actual usage and that most valid observations involve dosages in the upper range of the dosage–mortality curve. In the present case this would involve the 0.1 to 1.0% range in dosage giving 42% to 100% mortality. At these concentrations most of the eggs continued development to the stage when ChE appeared (stage E). It seems likely that the lowest dosage at which individual eggs could be killed would

TABLE I

Percentages of *Pieris brassicae* eggs developing normally to each stage after treatment with aqueous TEPP (Potter *et al.* (6))

Age in days	Stage of development	Approximate concentration of TEPP, %							Mean no. of eggs examined at each concn.
		Control	0.1	0.3	1.0	3.0	6.0	12.0	
1–2	A	93	92	74	79	79	80	60	17
2–3	B	69	93	94	86	71	50	0	18
3–4	C	83	87	86	68	83	50	-	17
4–5	D	100	64	82	78	77	30	-	16
5–6	E	79	80	56	42	52	0	-	18
6–7	F	96	74	44	38	22	-	-	19
7–8	G	92	63	47	25	13	-	-	19
Hatching		91	58	17	0	0	-	-	85

[2]1/4 pt TEPP (40%) per 100 U. S. gal of water.

result in death at advanced stages. This suggests the possibility of two modes of action, the most sensitive being ChE inhibition and another being superimposed at higher concentrations which halts development before occurrence of ChE. Such a possibility must be reconciled with the smooth transition in symptoms which takes place as dosage is increased. An analogous situation might be depicted by dosage–mortality curves representing two toxicants having different modes of action. If the curves were parallel and some overlapping in dosage occurred the transition in kill by another mode of action would be gradual as in the data cited. With some other organophosphates much greater spread occurs between the dosage required to prevent development and that which just stops hatching. This ratio has been reported as greater than 100 for paraoxon and less than 30 for TEPP (12). Thus in the case of paraoxon complete kill would likely have been reached by dosages which just prevented hatch and far greater dosages required to involve the mode of action which prevents development. In a number of species parathion induces complete kill with all individuals reaching advanced stages. Presumably higher dosage levels could be reached which would induce kill in early stages.

The point raised earlier that the instability of TEPP implies toxic action soon after application is open to question. While some hydrolysis would likely occur it is possible that a portion of the toxicant would be stored in the lipid component of the egg. In this case as with peach tree borer eggs it seems that experimental results can best be reconciled by a scheme involving storage of the toxicant. This rather basic point has recently been under investigation at this laboratory. In these tests peach tree borer eggs were exposed to parathion vapor for the first 2 days of incubation. On the eighth day when hatch would normally occur, breis were prepared from the treated eggs and these were added to a convenient source of ChE, namely fly heads. Marked inhibition of ChE occurred indicating that an inhibitor derived from parathion had been retained throughout embryonic development.[3] The retention of high levels of inhibitor in embryos alive but "marked for death" gives rise to interesting speculation.

It seems likely that the same storage mechanism cited here might apply to TEPP, but with greater loss occurring in the interval from treatment to storage. This might account for the poor ovicidal effectiveness of TEPP as compared with its high anti-esterase activity. Certainly newly laid eggs killed with minimal dosages of TEPP follow a course of development similar to that observed with parathion treatment in which storage of the toxicant has been demonstrated.

Further, speculating on the possibility of other esterases being involved in ovicidal action, Potter (12) has pointed out that the enzymes hydrolyzing triacetin are much less susceptible to inhibition than some others. In addition the values cited for TEPP in inhibiting the enzyme hydrolyzing phenyl acetate are relatively high (8). While the evidence available does not permit valid comparisons, it seems likely that, of the various esterases known to be present, ChE is the most sensitive to inhibition by organophosphates.

[3]Unpublished.

If a vital role is attributed to the other esterases despite the absence of supporting evidence, the findings of Potter *et al.* (6) might be reconciled as follows: Organophosphate compounds inhibit several vital esterases. The one most sensitive to inhibition is ChE which occurs only in late stages of embryonic development. Minimal lethal dosages will therefore involve this enzyme if the inhibitor is readily stored, and death will then occur in late stages of development. Higher dosages will involve esterases present at all stages of development thus accounting for death in early stages. It is equally plausible that higher dosages involve modes of action in which esterases are not involved.

Based on the respiratory rate of treated eggs, Staudenmayer (29) concluded that E600 (paraoxon), unlike some other organophosphates, induces toxicity before the appearance of ChE, but this conclusion hardly seems justified from the evidence cited.

In vivo inhibition studies are undergoing reappraisal based on the recent findings of Asperen (30). These results suggested that in vivo inhibition values previously cited are likely higher than those actually occurring in the intact organism. This discrepancy stems from the additional inhibition occurring when free inhibitor is released on homogenizing tissue. Lower inhibition values were obtained when ChE was protected against further inhibition by the addition of substrate to the homogenization saline. Still more striking was the disclosure that higher inhibition values occurred for ali-esterase than ChE, suggesting the former as the vital enzyme in organophosphate poisoning. Important as this finding is, the case for ali-esterase remains weak pending evidence that it plays a vital role.

While these findings may alter inhibition values previously ascribed they do not yet invalidate the significance of ChE inhibition. The evidence focuses attention on whether the high inhibition values previously cited are required for toxic action. It has been commonly recognized that the conventional methods of ChE assay measured gross activity in which the inhibition level in some restricted but vital area might be obscured. The possibility of such artifacts highlights the need for more precise methods for ChE assay. A significant step in this direction is the histochemical method recently employed by Winton *et al.* (31) for cellular localization of ChE in the nervous tissue of insects.

From the foregoing it appears that ovicidal action of organophosphates results from the inhibition of an esterase. Whether this esterase is ChE of the nervous system, ali-esterase whose function is unknown, or still another cannot be clearly established from existing evidence. The salient points favoring ChE inhibition are:

1. Early ovicidal treatment results in continued development to the stage when ChE and ACh occur.
2. The inhibitor may be stored prior to the incidence of ChE.
3. Differential susceptibility in two species coincides with in vivo ChE inhibition.
4. ChE inhibition results in abnormally high levels of ACh.
5. ACh and ChE are components of a known vital physiological system.

The essential points favoring the inhibition of other esterases are:

1. Eggs can be killed in early stages.
2. Other esterases are present in all stages.
3. These esterases are inhibitable by certain organophosphates.
4. The degree of inhibition of other esterases may be higher than that for ChE.

Taken alone either of these alternatives appears reasonable and to decide between them involves a certain degree of speculation. The weight of evidence appears to the writers to still favor ChE inhibition. In choosing this alternative, weight is accorded the fact that ACh and ChE are assigned roles in a vital physiological system. A number of features tend to obscure other evidence. Chief among these is the retention of the toxicant. If this did not occur, early treatment would presumably be ineffective. On the contrary, effectiveness of early treatment occurs later when the ACh–ChE system is assembled.

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SIGNIFICANCE OF PLANT METABOLITES OF INSECTICIDES[1]

E. Y. Spencer

Those metabolites of insecticides produced by plants which are of significance are centered largely around the organophosphorus insecticides with one main exception. The unsaturated chlorinated hydrocarbon aldrin has been shown to be oxidized in carrots as well as in alfalfa to the more toxic compound, dieldrin (1). Thus methods of analysis must be such that the two compounds can be estimated separately to avoid obtaining misleading residue data. The residue of the relatively stable chlorinated hydrocarbons is largely reduced by the microflora in the soil, the rate depending on the type of soil, moisture content, temperature, etc.

Activation and Degradation

The organophosphorus insecticides as a group are greatly affected by metabolism in the plant with a twofold result (2). In some instances a relatively stable compound is converted to a more labile and much more toxic material. Thus besides being more toxic it is also more readily hydrolyzed and therefore the residue problem is solved. Many of the compounds that are activated possess the valuable property of being systemic; that is, they may be applied at one spot or part of the plant and are sufficiently stable to be translocated throughout the plant and render the whole of it toxic for a period of time. The practical advantages of this are obvious. The hazards are equally obvious when we deal with edible crops. Thus the rate of metabolism and ultimate degradation in the plant are important for each compound if the appropriate time for application of the material is to be determined so that no residue which may be a hazard to health remains at the time of harvesting. The extent of degradation will also depend on the rate of growth of the plant and therefore on the season. Since this degradation may vary from 3 to 5 weeks with some systemic organophosphorus insecticides to 2 days with a recently developed one, and the mode of activation also varies, it would seem advisable to discuss several examples.

The organophosphorus insecticides are inhibitors of certain enzymes, notably those possessing carboxylic esterase activity. They are usually associated with the cholinesterases and thus the extent of cholinesterase inhibition is often a measure of their in vitro activity. Although there is a high correlation between anti-cholinesterase activity and toxicity it is not surprising that there are a number of anomalies since toxicity is dependent on several factors. However, the anti-cholinesterase property has been very useful in estimating the

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activity of most of the metabolites of organophosphorus insecticides. Rate of hydrolysis has also correlated well with toxicity with notable exceptions.

Activation by Oxidation

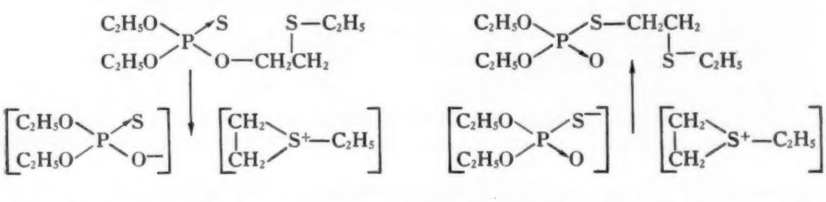
One of the earlier organophosphorus insecticides, a phosphoramide, schradan (octamethylpyrophosphoramide), is relatively stable to hydrolysis. It has a half life at pH 7 of 10 years by contrast to that of a pyrophosphate, TEPP (tetraethylpyrophosphate), of 6½ hours and of a thionophosphate, parathion (O,O-dimethyl O-*p*-nitrophenyl phosphorothionate), of 120 days. Its anticholinesterase activity as indicated by the pI_{50} value (negative logarithm of molar concentration required to inhibit 50% of the cholinesterase activity) is less than 2 while that of TEPP is about 8, that is, TEPP is one million times as active. Despite this low anticholinesterase activity and resistance to hydrolysis, schradan is very toxic to mammals and certain insects and is degraded in the plant over a 3- to 5-week period, depending on the rate of growth of the plant. This initial stability of schradan enables it to be translocated throughout the plant and makes it a systemic poison. Subsequent conversion in the plant, insect, or mammal to an active metabolite renders it toxic and at the same time accelerates its decomposition and so reduces toxic residues to a minimum in a limited time. This mode of activation has been studied by many (2). Although this activation has been shown to be an oxidation and although the active material from the various sources is apparently identical, only recently has the mechanism been elucidated and some anomalies explained (3). The proposed mechanism is an oxidation at one of the nitrogens, possibly yielding a transient intermediate which immediately rearranges to the methylol derivative. This is the reactive component with an activity approaching that of TEPP and a half life at pH 8 and 25° C of about 60 minutes by comparison with that of schradan itself of several years. This active material is degraded by phosphorylating certain enzymes such as the cholinesterases and by hydrolysis as well as by the splitting off of formaldehyde yielding a stable material. The actual amount of active material present in the plant is insufficient to account for the toxicity to mammals and to some insects since it is rapidly hydrolyzed and therefore contributes largely only to the degradation of the insecticide.

Another group of organophosphorus insecticides which require oxidation to an active derivative are the thionophosphates. For example, parathion has a half life of 120 days at pH 7 and yet has a relatively short life in growing plants of only a few days. Pure parathion has a very low anticholinesterase activity and is activated by oxidation of the thiono sulphur to the oxygen derivative, paraoxon, which is as reactive as TEPP. Thus the accelerated degradation in the plant is due to this oxidation together possibly with some hydrolysis by phosphatases.

The thiophosphates containing thioether substituents produce a complex mixture when metabolized by oxidation. In addition there is isomerization from the thiono to the thiolo derivative. For example, in demeton (a mixture

of thiono and thiolo isomers of O,O-diethylethylthioethylphosphorothioate), the thiono isomer has a much lower water solubility and is less reactive than the thiolo isomer. However, besides the oxidation of the thiono sulphur similar to parathion there is in addition the possibility of oxidation at the mercapto sulphur to the sulphoxide and further to the sulphone. Thus there are theoretically seven oxidation products (4), which are listed in Table I. It has been shown that the thiol isomer is more rapidly translocated and that oxidation to the sulphoxide occurs rapidly with both isomers yielding more reactive intermediates. Subsequently at a lower rate, comparable with the rate of oxidation of the thiono sulphur, the sulphoxides of both isomers are formed. Since the thiono sulphoxide isomer has two possible further pathways to be metabolized by contrast with only one for the thiol sulphoxide isomer it is not surprising to find that the latter persists for almost twice as long in the plant.

Another possible factor contributing to this more rapid loss is the isomerization of the thiono isomer via an ionic sulphonium intermediate to the thiol isomer shown by the following proposed mechanism (5):



Examination of Table I shows that some phosphates formed will be rapidly hydrolyzed and not contribute to systemic activity while oxidation to the

TABLE I

		Fly brain ChE inhibition, pI_{50}	Mole % hydrol., pH 7.9, 37° C, 60 min
Thiono isomer	$(C_2H_5O)_2P(S)OC_2H_4SC_2H_5$	3.7	9.4
Thiolo isomer	$(C_2H_5O)_2P(O)SC_2H_4SC_2H_5$	5.5	19.6
Phosphate	$(C_2H_5O)_2P(O)OC_2H_4SC_2H_5$	7.6	39
Thionosulphoxide	$(C_2H_5O)_2P(S)OC_2H_4S(O)C_2H_5$	5.5	6.2
Thiolosulphoxide	$(C_2H_4O)_2P(O)SC_2H_4S(O)C_2H_5$	5.8	1.6
Sulphoxide phosphate	$(C_2H_5O)_2P(O)OC_2H_4S(O)C_2H_5$	6.0	13.7
Sulphone phosphate	$(C_2H_5O)_2P(O)C_2H_4S(O)(O)C_2H_5$	6.9	—
Thiolo sulphone	$(C_2H_5O)_2P(O)SC_2H_4S(O)(O)C_2H_5$	6.2	9.7
Thiono sulphone	$(C_2H_5O)_2P(S)OC_2H_4S(O)(O)C_2H_5$	6.1	10.1

sulphoxide and sulphone enhances the anticholinesterase activity (6). Thus the metabolism of the demeton isomers by the plant increases the toxicity and ensures low toxic residues at the end of a month in the edible crops examined.

Two more recent thiophosphates are Dithio-Systox (O,O-diethyl S-ethyl-2-mercaptoethyl phosphorodithioate) and the closely related Thimet (O,O-diethyl S-methyl-mercaptomethyl phosphorodithioate). By contrast, no isomerization

as with the thiono to thiol isomer of demeton can occur. Both are less active than demeton as would be expected from the dithioate structure and require activation in the plant. As with demeton, oxidation to the sulphoxide takes place readily with subsequent slower oxidation to the sulphone and oxidation of the thiono sulphur yielding five possible isomers. As oxidation proceeds the anticholinesterase activity increases as shown in Table II (7). This metabolism has been followed by partitioning P^{32}-labelled starting material between chloroform and water (8). The relative rates of oxidation and disappearance of

TABLE II

	R_f	Fly head ChE inhibition, $I_{50}M$
Dithio-Systox series		
$(C_2H_5O)_2P(S)SCH_2CH_2SC_2H_5$	0.99	$>1 \times 10^{-4}$
$(C_2H_5O)_2P(S)SCH_2CH_2S(O)C_2H_5$	0.18	7×10^{-5}
$(C_2H_5O)_2P(S)SCH_2CH_2S(O)(O)C_2H_5$	0.64	3.5×10^{-6}
$(C_2H_5O)_2P(O)SCH_2CH_2SC_2H_5$	0.80	3.5×10^{-6}
$(C_2H_5O)_2P(O)SCH_2CH_2S(O)C_2H_5$	0.00–0.04	1.5×10^{-6}
$(C_2H_5O)_2P(O)SCH_2CH_2S(O)(O)C_2H_5$	0.03–0.08	6.0×10^{-7}
Thimet series		
$(C_2H_5O)_2P(S)SCH_2SC_2H_5$	0.99	2.5×10^{-5}
$(C_2H_5O)_2P(S)SCH_2S(O)C_2H_5$	0.31	3.7×10^{-6}
$(C_2H_5O)_2P(S)SCH_2S(O)(O)C_2H_5$	0.66	4×10^{-8}
$(C_2H_5O)_2P(O)SCH_2SC_2H_5$	0.53	5×10^{-7}
$(C_2H_5O)_2P(O)SCH_2S(O)C_2H_5$	0.0	4×10^{-7}
$(C_2H_5O)_2P(O)SCH_2S(O)(O)C_2H_5$	0.05	1×10^{-7}

intermediates can be readily followed over the 15-day period. The initial oxidation of Thimet is slower as would be expected from the closer proximity of the mercapto sulphur to the phosphorus but, once oxidized, activity and decomposition is slightly greater. Where the starting material has been applied to alfalfa seeds by contrast with the cotton plant above, the appearance of metabolites is slightly delayed but the most active material as well as the starting material disappears within 15 days.

All of the compounds discussed except the phosphates and phosphorthiolates require oxidation in their metabolism to more active compounds with simultaneous accelerated degradation to non-toxic residues. The residual toxicity remains between 3 and 5 weeks depending on the compound and the type of plant.

Non-oxidative Degradation

Another systemic organophosphorus compound, but with a very short life in the plant of only 2 days, has recently been introduced (9). It is a vinyl phosphate (O,O-dimethyl O-2-carbomethoxy-1-methyl vinyl phosphate), Phosdrin. This material is systemic in its action but by contrast does not require activation. Being a vinyl phosphate it exists as geometrical isomers. The alpha or *cis* isomer is less water-soluble but 100 times more toxic to mice than the beta or *trans* isomer. Their difference in toxicity to flies is not as great and to mites, even less. The property of particular interest as far as metabolism in the plant is concerned is that besides the 'normal' degradation to dimethyl

phosphate, preliminary experiments also indicate some hydrolysis of the carboxymethyl ester as well as some monodemethylation of the alkyl phosphate (10).

The alkaline hydrolysis of the two geometrical isomers has been shown to follow different pathways (11). The *trans* isomer yields largely dimethyl phosphate with a trace of monodemethylated *trans* Phosdrin. The *cis* isomer on the other hand gives a more complex mixture containing besides dimethyl phosphate, appreciable amounts of the free acid from the hydrolysis of the carboxymethyl ester and monodemethylated *cis* Phosdrin.

Another recent organophosphorus insecticide exhibiting systemic properties is a sort of nitrogen analogue of demeton, namely amiton (O,O-diethyl S-2-diethylaminoethyl phosphorothiolate). It requires no activation and, possibly partly due to its similarity in spatial configuration to acetyl choline, has high toxicity. The presence of nitrogen in such a position renders it basic and able to form salts. In addition the thiono isomer readily isomerizes to the thiol isomer similar to demeton and presumably via a cyclic imonium ion (12). The salt form of amiton is translocated more slowly than the free base on stem application, suggesting the influence of lipoid solubility on penetration. Toxic residues fall to the same low level during 4 weeks as with demeton but no metabolic intermediates have been detected. Thus unless contrary evidence is found this systemic insecticide differs radically from most others discussed in apparently not requiring the plant to assist in its activation and subsequent degradation.

Selective Toxicity

As an indication of the trend in the development of new insecticides, the withdrawal of amiton because of its very great toxicity and difficulty of handling without taking undue risks is of note.

Of particular interest are those organophosphorus insecticides that have a relatively low mammalian toxicity such as malathion (O,O-dimethyl S-1,2-biscarbethoxyethyl phosphorodithioate) compared to their insecticidal activity. Since the plant does not appear to play a significant part in this selective toxicity, further discussion will be limited to merely mentioning that this differential toxicity may be due to degrading enzymes in mammals that are absent in insects (13). A recent addition of an animal systemic foliar insecticide Rogor (Montecatini) or Dimethoate (American Cyanamid), O,O-dimethyl S(N-methylcarbamoylmethyl)phosphorodithioate — $(MeO)_2P(S)SCH_2C(O)NHMe$, presents further problems in identification and characterization of the several degradation products possible. Besides the usual oxidation of the thiono sulphur to the more reactive oxygen analogue there is also monodemethylation of the phosphate and amide hydrolysis—the former predominating in plants, the latter in mammals (14). As the properties of these compounds are explored and with the greater knowledge of the mode of action the apparent anomalies that have arisen are beginning to be explained.

In summary, metabolites of pesticides in plants are important largely in converting the material to more reactive and therefore usually more toxic

intermediates. These are confined almost entirely to certain organophosphorus insecticides. This in turn ensures the more rapid breakdown and resultant low toxic residues. In some, the activation is slow so that the active material is rapidly hydrolyzed and therefore it is largely the unoxidized initial material ingested by the insect and activated by it which is toxic. The conversion in the plant merely assures a low residue. In others, the plant is the essential activator converting the compounds rapidly to materials with high insecticidal activity or directly to innocuous degradation products. In a few instances the initial material itself is highly toxic and is detoxified by hydrolysis in the plant.

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TRENDS IN INSECTICIDAL CHEMICALS: A SUMMARY*

HUBERT MARTIN

Empirical hit-or-miss trial has proved a highly successful method of uncovering insecticidal properties, particularly among the organophosphate and cyclodiene compounds. Few would, 10 years ago, have prophesied that this method would have revealed organophosphates safe enough for internal use in cattle against such pests as the warble fly. The biochemist and physiologist have as yet had little information by which to direct the search except in most general terms, but their influence is now extending at a fast pace. O'Brien discussed yesterday his hypotheses as a means of achieving differential toxicity to arthropods and to mammals, ideas which have already led him to a series of insecticidal organophosphates of low hazard to man. But hitherto the direction given by the chemist has been non-specific, operating more towards the purposeful manipulation of solubility relationships to ensure the mobilization of the toxicant at its site of biological activity. Among the chemical tricks for extending the likely range of chemical candidates is that which relies on the dependency of toxicity on molecular structure permitting the replacement of a particular grouping by one of similar dimensions, e.g., the replacement of chlorine by methyl or methoxy groups. This device, illustrated by DDT and methoxychlor, has so far been more frequently exploited among the herbicides, e.g., trichloroacetic acid and dalapon. Of the molecular shape hypotheses, a familiar example is that of membrane disruption used by Mullins to account for insecticidal activity among the chlorinated hydrocarbons. But such hypotheses will require extension to embrace the newly described isodrin derivative with its curious bird-cage structure, or the dimer of hexachlorocyclopentadiene with its cube structure, provided that the insecticidal properties claimed in the respective patents (U.S.P. 2,714,617 and re-issue 24,435) prove of a high order.

The second example of a chemical device is the replacement of hydrogen by the similarly sized fluorine atom whereby those hydrogens involved in the reactions of the biochemical lesion may be detected. This method was brilliantly used for the examination of the carcinogenicity of butter yellow (4-dimethylaminoazobenzene) by which the Millers established the significance of the hydrogens at the 2- and 6- positions of the aminobenzene.

Chance will eventually give way to reason in the search for new insecticides as our knowledge of the mechanisms underlying insect behavior grows. Of the several fields for development, two seem outstanding. The first is the study of the role of hormones, particularly in insect metamorphosis. The second field of work, the reasons why many insect pests have a limited host range, may also reveal chemical methods for avoiding their attack on the host plant more subtle than those in current use.

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NOTES TO CONTRIBUTORS

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